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Automated sample preparation for the determination of budesonide in plasma samples by liquid chromatography and tandem mass spectrometry

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Abstract

An automated bioanalytical method for the determination of the glucocorticosteroid drug budesonide in plasma samples at p*M* levels was investigated. The method was built using three separate automated analytical steps with manual transfer of samples between them. In the first step, a Tecan RSP150 (Genesis) pipetting robot was used to transfer 1 ml of centrifuged plasma samples and deuterated budesonide internal standard solutions into tubes and to homogenise the resulting admixture. In the second step, a solid-phase extraction was performed using an ASPEC Xli (Gilson) with 100 mg Isolute C_{18} columns. In order to avoid conventional time-consuming evaporation and reconstitution steps, the solid-phase extraction was coupled on-line to a trace enrichment system for further purification and concentration of the sample extracts. The concentrated samples were eluted in 300 μl ethanol into injection vials, which were capped and transferred to the autosampler in the detection system. In the third step, the pre-treated samples were chromatographed in a gradient LC system and detected using a tandem MS system (Finnigan TSQ 7000), with an atmospheric pressure chemical ionisation interface. The described Analytical System consisting of one Tecan robot, two ASPEC systems and one LC–MS–MS system may analyse up to about 800 samples a week with less routine work for the analyst. The concentration range studied was 15 to 2500 p*M* in 1 ml spiked plasma samples and the limit of quantitation for the described method was determined as 15 p*M*, as defined by accuracy and precision better than 20%.

Keywords: Automation; Sample preparation; Budesonide; Corticosteroids

1. Introduction

In the development of pharmaceutical drugs, there is a demand for bioanalytical methods possessing high sensitivity, selectivity and sample throughput.

As the plasma samples enter the bioanalytical laboratory a series of steps are performed before a final analytical result is obtained. In most laboratories the sample preparation and the clean-up steps are still performed manually [1]. Only the chromatographic and detection step is always run automatically, i.e., using an autosampler connected on-line with the chromatographic system and the detector [1].

Different techniques for automation of sample clean-up procedures have been developed. In automated sample handling systems such as the Prospect (Spark Holland), PrepStation (Hewlett-Packard), the Rapid trace (Zymark) and the ASPEC Xli, (Gilson), the solid-phase extraction (SPE) procedure is automated, on- or off-line [2–6]. Direct injection of biological samples, using columns with restricted access material (RAM), is another technique in progress [7–9]. However, this technique is still not straightforward to use for large numbers of samples

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and large plasma volume injections without time-consuming column rinsing procedures between each sample [10]. If the analyte is strongly protein-bound it may also require some off-line dilution to dissociate the analyte from the protein [11].

If increased productivity is desired, not only the sample clean-up steps need to be automated, but also the first preparation steps [2]. The transfer of precise volumes of samples into vials and addition of reagents, such as internal standard, can effectively be performed using pipetting robots, such as the Beckman Biomek 2000, the Packard Multiprobe, the Tecan Genesis RSP 150, or several others available on the market [12]. In this way tedious routine work and occupational injuries in the laboratory may also be avoided [13].

We have developed a method where the sample preparation, clean-up and the separation and detection steps all have been automated in separate workstations using commercially available equipment. Division of the equipment into separate workstations keeps the system complexity down, which decreases the risk of system failure. For the analyst the flexibility of the laboratory work is increased, as it becomes easier to schedule various activities on an everyday basis. The glucocorticosteroid budesonide was used as a model compound in order to illustrate this work.

2. Experimental

2.1. Instrumentation

A Genesis Tecan RSP 150 pipetting robot (Tecan, Hombrechtikon, Switzerland) was used to add internal standard (I.S.) and to dispense the plasma samples.

The SPE procedure was carried out using a Gilson ASPEC Xli (Gilson, Middleton, WI, USA) equipped with three bottles containing methanol, water and 70% ethanol in water, respectively. The separate reservoir solution used was 35% methanol in water. Isolute C_{18} MF, 100 mg with a 1-ml reservoir (Sorbent, Frölunda, Sweden) was used as the solid-phase in the extraction. The software for the SPE procedure was written at the department, in Turbo Pascal using Gilson 719 Sampler Manager Software 2.00.

The eluate was injected, by the ASPEC, into a trace enrichment flow system. The flow system, shown in Fig. 1, consisted of four Pharmacia LC pumps, Models 2248 and 2150, (Pharmacia Biotechnology, Bromma, Sweden), two six-port Valco valves (Valco Instruments, Houston, TX, USA), carrying the pre-column (Kromasil NH_2, 5 μm, 10× 4.6 mm, Scantec, Sweden) and the enrichment column (Kromasil C_{18}, 5 μm, 10×4.6 mm, Scantec). A Gilson FC 204 fraction collector was used for collection of 300-μl samples, using a Gilson 832 temperature regulator kept at 4°C. The valves and the fraction collector were controlled by Access*Chrom, (PE Nelson Systems, Cupertino, CA, USA), using A/D converters PE Nelson, 941 Intelligent Interface from PE Nelson Systems.

An LC gradient system, shown in Fig. 2, was used in combination with the MS–MS detector. Two Pharmacia 2248 pumps were controlled by a Pharmacia LCC 2252 control unit (Pharmacia Biotechnology). A volume of 225 μl of each sample was injected by a Spectra-Physics AS 3000 autosampler (Spectra-Physics Analytical, San Jose, CA, USA) into a flow of ethanol–water (95:5) containing 0.2% acetic acid. After an on-line dilution with water–ethanol (5:95) containing 0.2% acetic acid, the sample plug was mixed using two Visco mixers (Lee,

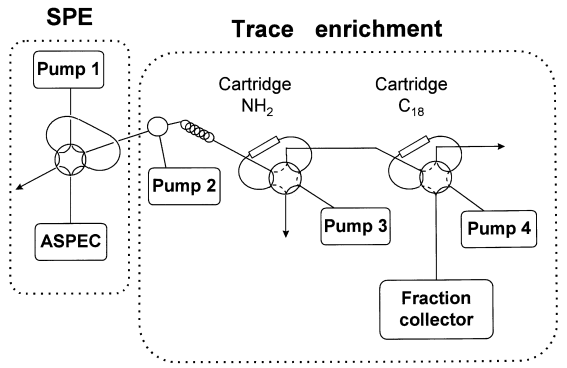

Fig. 1. The sample clean-up step. The eluted analyte from the ASPEC is carried from the injection loop in 70% ethanol in water, diluted on-line with 5% ethanol in water, mixed and then trapped on the enrichment cartridge. While the elution of the enriched analyte is carried out in 95% ethanol, the NH_2-cartridge is rinsed with 99% ethanol.

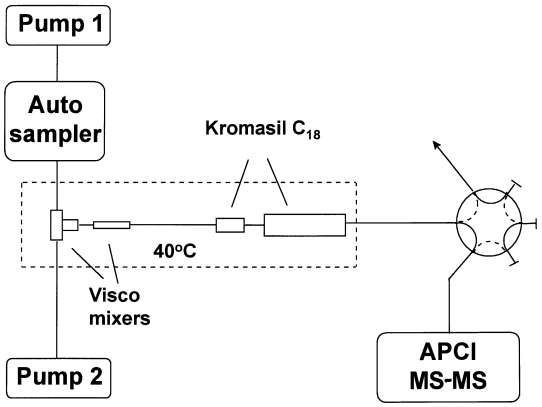

Fig. 2. The LC system for the detection. The samples were introduced in ethanol–water (95:5) containing 0.2% HAc and diluted on-line with water–ethanol (5:95) containing 0.2% HAc.

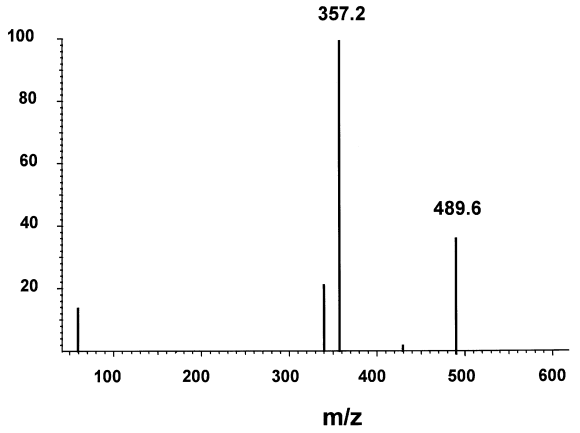

Fig. 3. The mass spectrum of budesonide. The detection was performed by negative single ion monitoring of the m/z 357.2 fragment of the m/z 489.6 acetate adduct of budesonide formed in the APCI interface.

Westbrook, CT, USA), one T-mixer with an internal volume of 10 μl, and one in-line mixer with an internal volume of 250 μl, at a total flow-rate of 1.0 ml/min, before entering the pre-column (Kromasil C_{18}, 5 μm, 10×2.1 mm, Chromtech, Hägersten, Sweden) and the analytical column (Kromasil C_{18}, 5 μm, 50×2.1 mm, Chromtech). Columns and mixers were thermostatted at 40°C with a Jones Chromatography Column Chiller Model 7955 (Sorbent). The LC gradient, shown in Table 1, was used. A Valco six-port valve was used to switch the LC flow to waste when no data collection was taking place, in order to minimise contamination of the MS–MS system.

A Finnigan TSQ7000 mass spectrometer (Finnigan MAT, San Jose, CA, USA) was used for the detection, with an atmospheric pressure chemical ionisation (APCI) interface. The detection was performed by negative single ion monitoring of the m/z 357.2 fragment of the m/z 489.6 acetate adduct of budesonide formed in the APCI interface, Fig. 3. The vaporiser temperature was 450°C, the sheath gas pressure 22 p.s.i. (1 p.s.i.=6894.76 Pa) and the corona current 5 μA. The capillary temperature was 155°C and the collision potential 20 V using argon at 2 mTorr as a collision gas (1 Torr=133.322 Pa).

Table 1
The LC gradient used in the LC system for the liquid chromatography and detection

Time (min)	B (%)	Ethanol (%)
0	16	19
2.5	16	19
4.3	65	64
4.5	65	64
4.6	100	95
5.0	100	95
5.1	16	19

2.2. Materials

Budesonide and internal standard [$^2H_8$]budesonide, Fig. 4, were synthesised at Astra Draco. Human blank blood plasma was obtained in sodium-heparinised glass tubes, centrifuged at 1300 *g* for 10 min and frozen at −70°C. The ethanol (95%

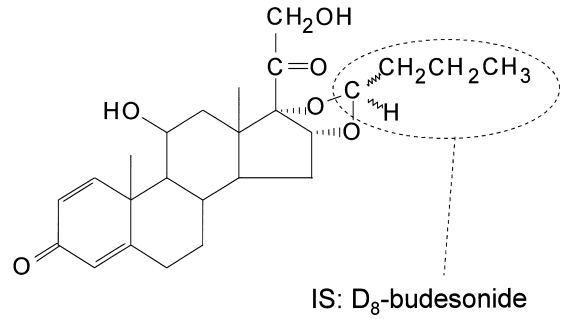

Fig. 4. The structure of budesonide. In the internal standard the hydrogen atoms in the acetale group have been exchanged for deuterium atoms.

and 99.5%) came from Kemetyl (Stockholm, Sweden), methanol (HPLC-grade) was purchased from Fisons (Malmö, Sweden). Formic acid (analytical-reagent grade) was from Merck (Darmstadt, Germany), the acetic acid, 99.7% ACS reagent, from Aldrich (Stockholm, Sweden) and the water used was purified to TOC<4 ppb (Milli-Q, Millipore, Gothenburg, Sweden).

2.3. Preparation of plasma standards and plasma quality controls (QCs)

Human blank plasma was thawed and centrifuged at 250 *g* for 15 min, to eliminate precipitates. The plasma standards, were spiked with budesonide, dissolved in ethanol–water (65:35) to concentrations of 15.0, 50.0, 100, 500, 1000 and 2500 p*M* and were gently stirred for 30 min at ambient temperature before freezing. The corresponding plasma standard solutions were divided into 1.3-ml aliquots in polypropylene tubes with screw caps (2 ml), and frozen at −70°C. Nine QC samples at each concentration were used for determining the accuracy and precision of the method. The QC samples were prepared in the same way to concentrations of 15.0, 25.0, 50.0, 100, 250, 500, 1000, 2100 and 2500 p*M*.

3. The analysis procedure

The analysis procedure was performed in three physically separated work stations.

3.1. Dispensing of plasma standard samples and QC samples

Duplicate plasma standard samples and the nine QC samples were thawed at room temperature and centrifuged at 250 *g* for 15 min, to eliminate precipitates. The standard samples and QC samples were placed in the Tecan Genesis pipetting robot and 1.00 ml was dispensed into polypropylene tubes (3 ml), using disposable tips. Prior to this step 50 μl of the internal standard solution ($[^2H_8]$budesonid dissolved in 65% ethanol in water) had been added by multidispensation. The resulting solution was mixed with an aspiration and a redispensation procedure at a high flow-rate. The number of samples in a dispensation series was 108, i.e., the number of samples possible to load in an ASPEC using 1-ml cartridges.

3.2. Solid-phase extraction and trace enrichment

The SPE cartridges were conditioned in batch with 0.6 ml methanol followed with 0.6 ml water with 0.1 ml air in between. The extraction procedure was performed in sequence. Budesonide is stable in plasma at ambient temperature for the time period needed to process all samples. The SPE procedure consisted of an addition of a 1 ml plasma sample followed by 0.4 ml air. The washing of the cartridge was performed with 1.5 ml water and 1.0 ml 35% methanol in water with 0.1 ml air in between. The elution was performed with 0.5 ml 70% of ethanol in water followed with 0.75 ml air. In sequence with the 0.5 ml eluate, 0.3 ml of 70% ethanol in water (with a 0.4 ml air segment in between) was injected into the injection loop to the trace enrichment system.

The enrichment procedure was performed according to the following scheme. A solution of 70% ethanol in water (pump 1, Fig. 1) was used to carry the eluate from the injection loop in the ASPEC into the trace enrichment system. The ethanol concentration in the sample was diluted to 25% ethanol in water by mixing with 5% ethanol in water (pump 2, Fig. 1), giving a total flow-rate of 2 ml/min. The enriched samples were eluted from the enrichment column with 95% ethanol in water at a flow-rate of 0.5 ml/min (pump 4, Fig. 1) directly into injection vials, which were capped and transferred to the LC–MS–MS system. At the same time the NH_2 cartridge pre-column was washed by back-flushing between each sample, with 99% ethanol in water at a flow-rate of 2 ml/min (pump 3, Fig. 1).

The trace enrichment system and the SPE worked concurrently, i.e., while sample 1 was enriched, sample 2 was simultaneously extracted on the ASPEC.

3.3. Liquid chromatography and MS–MS detection

Before the enriched extracts were chromatographed and quantified in the LC–MS–MS system, Fig. 2, the system suitability was checked by in-

jections of reference solutions of 80 pmol/l budesonide and 8.9 nmol/l of internal standard in 95% ethanol in water.

The quantification was performed using standard polynomial quadratic regression weighted vs. $1/x^2$.

4. Results and discussion

4.1. Preparation step

The pipetting robot was able to prepare 108 samples in 30 min. By using an automated procedure, better precision can be obtained in a continuous routine situation than achieved in manual pipetting. Plasma pipetting using the Tecan gave a relative standard deviation (R.S.D.) of around 0.2% ($n=20$), while manual pipetting of plasma normally yielded a R.S.D. of around 0.6%. Ethanol solutions are by experience hard to pipette with precision, still the Tecan robot gave a R.S.D. of around 0.5% ($n=20$) when pipetting 50 μl of the 65% ethanol in water solution. Another benefit is that GLP documentation is improved when using a robot, as data are gathered in the computer on the success of each step in the pipetting procedure. If desirable the Tecan robot used may be equipped with a bar code reader, which would further improve GLP documentation.

4.2. Sample clean-up

4.2.1. Optimisation of the SPE

Automation of SPE with the ASPEC in the n*M* to p*M* concentration range, requires extraordinary attention to dispensing procedures in order to optimise various factors such as carry-over between samples and recovery in each operation and at the same time maintain time-efficiency [14,15]. When designing the SPE procedures all movements of the needle should thus be time-efficient in order to decrease the treatment time for each sample.

Due to the way the ASPEC is designed, all liquid volumes needed in the SPE procedure are aspirated in the needle and aspiration coil. It is therefore important to include efficient rinsing steps to avoid contamination between samples. Between each step the needle and coil were therefore washed with the reservoir solvent.

However, in terms of recovery it is also important not to lose too much of the analyte in these rinsing steps. An effective way to avoid this is to aspirate liquid segments sequentially, separated with small segments of air to minimise mixing between the aspirated liquid volumes, before they are dispensed on the SPE cartridge.

When loading the plasma sample on the SPE cartridge, the water segment for the wash was aspirated prior to the sample and dispensed directly after the sample. The second wash solution consisted of reservoir solution, which was dispensed onto the cartridge in sequence with the water wash. In this way the water and methanol segments also rinsed the coil from remaining analyte and transported it to the SPE cartridge, leading to an increased recovery.

In the elution, the extracted analyte was eluted in a small volume of 70% ethanol in water, sufficient to displace all of the sample.

In the injection, a wash zone of 70% ethanol in water was aspirated prior to the eluate. A slightly larger volume than the eluate was then injected into the injection loop of the trace enrichment system. The ethanol solution remaining in the coil and a volume of reservoir solvent was then used to rinse the injection port.

These procedures were found essential in order to avoid contamination between samples and still get a good recovery of the method.

4.2.2. Trace enrichment system

Since the trace enrichment and the SPE worked in a concurrent mode, it was important to optimise the duration for both procedures. Insertion of an amine cartridge pre-column was previously found to give cleaner eluates from extracted plasma samples, and therefore it was used in this trace enrichment system. The ethanol concentration was adjusted to 25% in water to give efficient trapping of the analyte on the enrichment column with a minimum of band broadening in the pre-column.

The length of time needed to enrich the analyte, ''the enrichment window'', was studied by using plasma spiked with tritiated budesonide and collecting small fractions exiting the amine cartridge, Fig. 1. In Fig. 5A, it is shown that 5 min is sufficient for efficient enrichment of budesonide, since all of the

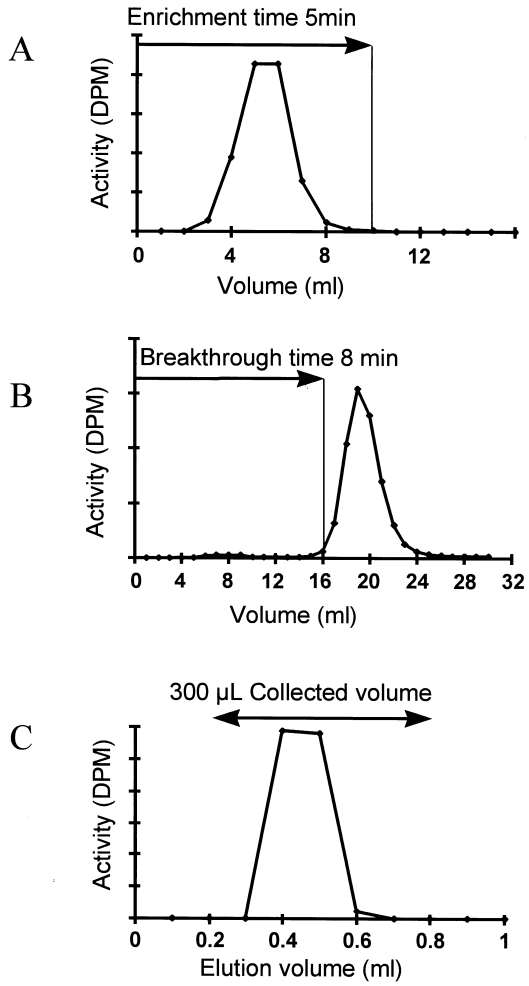

Fig. 5. The performance of the trace enrichment system: (A) shows the analyte exiting the amine cartridge and the time needed for quantitative enrichment; (B) shows the ''breakthrough volume'' of the analyte on the enrichment column is greater than 8 min; (C) shows the elution volume of the analyte on the enrichment column.

analyte will have passed the amine cartridge in that time.

The ''breakthrough volume'' of the enrichment cartridge, Fig. 1, was studied in the same way by collecting fractions exiting the enrichment cartridge, after injection of plasma spiked with tritiated budesonide. The chosen enrichment time of 5 min showed to be sufficient to avoid ''breakthrough'' and loss of analyte. Fig. 5B shows that more than 8 min are needed for the analyte to pass the enrichment column.

The ''elution profile'', i.e., the solvent volume needed to effectively elute the analyte from the enrichment cartridge, Fig. 1, was investigated analogously by collecting eluting small fractions of tritiated budesonide exiting the enrichment cartridge. As seen in Fig. 5C, a volume of 300 µl effectively collected all of the analyte.

In order to avoid uncontrolled evaporation of the ethanol, 95% in water in the collected extracts, the vials were kept in thermostatted cooling racks at 4°C.

4.3. Separation and detection

The samples from the trace enrichment step were eluted in 95% ethanol in water, they were injected using a 95% ethanol in water mobile phase to effectively minimise carry-over in the autosampler. In order to achieve peak-compression and a good chromatographic separation, the organic phase was mixed on-line with a low concentration of organic phase. The mixing was found to be quite efficient using the two Visco mixers described in Section 2.1.

In order to obtain an efficient aerosol formation and analyte ionisation in the APCI interface of the MS–MS system, a rather high mobile phase flow-rate was needed, 1 ml/min. Due to the high back pressure over the analytical column at this flow-rate, it was necessary to increase the column temperature to 40°C to decrease the viscosity of the mobile phase. The chromatographic behaviour was not negatively affected by the increased column temperature, and the expected column life-time was drastically increased.

The very high selectivity and sensitivity of MS–MS was essential to achieve good analytical data at the very low sample concentrations studied (down to fmol injected), even after the rigorous sample clean-up and LC performed. Fig. 6 shows three typical chromatograms from top to bottom. The top graph, shows a chromatogram of the internal standard [$^{2}H_{8}$]budesonide at 2000 p*M*. The middle graph shows a low concentration budesonide QC sample spiked to 15 p*M*. The bottom graph shows the background after injection of blank plasma sample.

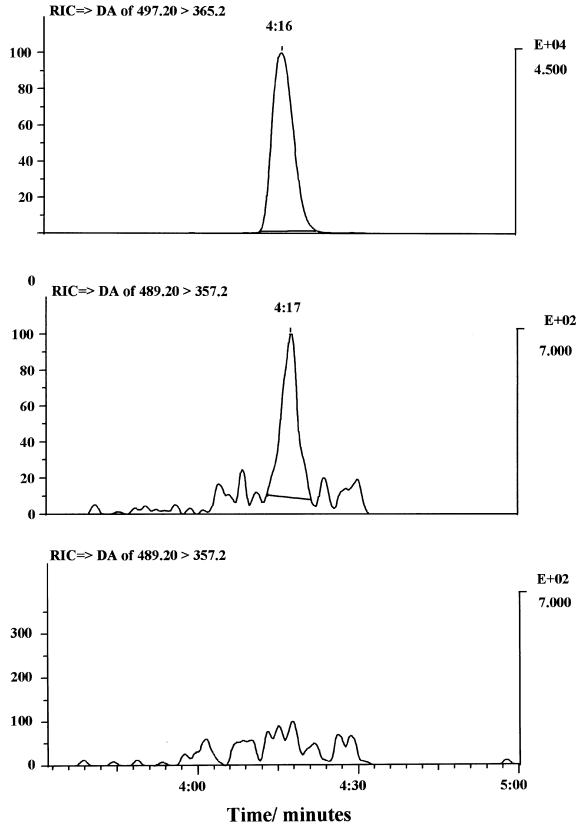

Fig. 6. Three typical chromatograms. The top graph, shows a chromatogram of the internal standard [$^2H_8$]budesonide at 2000 p*M*. The middle graph shows a low concentration budesonide QC sample spiked to 15 p*M*. The bottom graph shows the background after injection of blank plasma sample.

4.4. Method characteristics

The standard curve consisted of duplicate standard samples at each concentration level, which in the experiment described gave the equation of the curve: $y=64.3x^2+1046x+4.52$, $r=0.991$. The found accuracy and precision over the calibration range, 15 to 2500 p*M*, Table 2, were within the unofficially acceptable criteria for a bioanalytical method [16,17]. As the precision and accuracy was better than 20% the limit of quantification (LOQ) was set to 15 p*M* and the analytical range of the method to 15–2500 p*M* for 1-ml plasma samples. The relatively higher values of the accuracy at the low end, 15 and 25 p*M*, of the calibration curve were probably due to difficulties in fitting a non-linear signal response from the mass spectrometer to a polynomial evaluation of the calibration curve.

The total analyte recovery of the method was determined to be 81±2%, $n=8$ using radioactively labelled budesonide. The carry-over of analyte in the analytical system was found to be less than 0.2%. If a lower carry-over would be required, the presumptive source of contamination, i.e., the injection port on the ASPEC, may be rinsed more efficiently by several different procedures.

4.5. Assembly of the workstations and the analytical system

In order to get a balanced sample handling rate between the three separate workstations, several details have to be considered. The effective use of each set-up on a 24 h basis is important, as well as finding a good work situation for the analyst. In Table 3 the total number of samples possible to load each single set-up of equipment, the time needed for handling each sample and the handling time for the total number of samples is indicated. It is obvious that the Tecan is very fast, and that it in principle

Table 2
The found accuracy and precision over the calibration range for plasma QC samples spiked in the range 15 to 2500 p*M*

	Concentration of spiked QC samples (p*M*)								
	15	25	50	100	250	500	1000	2100	2500
Measured mean (p*M*)	17.8	29.2	48.5	96.9	248	514	986	2180	2520
Accuracy (%)	118	117	97.0	96.9	99.3	94.6	98.6	103	103
Precision (%)	12	12	6.6	5.7	6.1	3.4	2.5	3.3	0.8

$n=9$.

Table 3
The number of samples possible to load each set-up of equipment, the time needed for handling each sample and the total handling time for the process

Single set-ups	Max. No. of samples loadable	Time/sample (min)	Completion time (h)
Tecan RSP 150	230	0.28	1.1
ASPEC+enrichment	108	6.5	12
LC–MS–MS	120	5.1	10

could supply up to six ASPEC systems every day. The sample handling rate for the ASPEC and the LC–MS–MS systems are almost identical, but much slower than for the pipetting robot. There are many possible ways to combine the set-ups of equipment into workstations, depending on what factors are considered the most important for a specific situation.

In the analytical system described in this work, one Tecan pipetting robot was combined with two ASPEC trace enrichment set-ups and one LC–MS–MS system. In this way a balanced sample handling rate between the workstations and an efficient use of the most expensive piece of equipment, the MS–MS system was achieved. With an analytical system put together in this way, a work schedule according to the following, may be planned. Day 1 the manual preparations, the dispensing of the samples with the internal standard addition and the SPE with the trace enrichment are performed. Day 2 the samples are analysed in the LC–MS–MS system.

By running four analytical sequences like this, it is possible to analyse up to about 800 samples a week on a routine basis. This throughput potential by far exceeds the need for sample analysis in most situations, even taking into consideration service and maintenance of the equipment. The work of the analyst is concentrated on keeping the equipment in good condition, monitoring the performance and to examine and evaluate the analytical results.

5. Conclusions

We have found the analytical system consisting of three automated workstations to be very effective in handling large amounts of samples on a routine basis. The automated dispensing of sample and internal standard is very fast and has good accuracy and precision. It excludes the need of strenuous manual pipetting and also the variation in pipetting performance between analysts. The automated SPE with the on-line trace enrichment results in controlled extraction conditions compared to manual work. The evaporation and reconstitution commonly used after the SPE is eliminated, which means less manual interference and better time efficiency.

The separation of the analytical system into workstations makes it easier to work with the single pieces of equipment than for a large and complex on-line system. It is also easier to effectively use the expensive MS–MS equipment as seen as a laboratory resource allocation. As the analytical chemist will spend less time doing routine laboratory work, more time will be available for the development of new methods and technologies.

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References

[1] R.E. Majors, LC·GC 14 (1996) 754.
[2] R.E. Majors, LC·GC 13 (1995) 742.
[3] I. Ferrer, V. Pichon, M.C. Hennion, D. Barcelo, J. Chromatogr. A 777 (1997) 91.
[4] C. Molina, P. Grasso, E. Benefenati, D. Barcelo, J. Chromatogr. A 737 (1996) 47.
[5] A. Ceccato, P. Chiap, P. Hubert, J. Crommen, J. Chromatogr. B 698 (1997) 161.
[6] A.C. Hogenboom, P. Speksnijder, R.J. Vreeken, W.M.A. Niessen, U.A. Brinkman, J. Chromatogr. A 777 (1997) 81.

[7] A.J. Oosterkamp, H. Beth, M. Beth, K.K. Unger, U.R. Tjaden, J. van der Greef, J. Chromatogr. B 653 (1994) 55.
[8] R.E. Majors, K.S. Boos, C.H. Grimm, D. Lubda, G. Wieland, LC·GC 14 (1996) 554.
[9] R. Vanderhoeven, A.J.P. Hofte, M. Frenay, H. Irth, U. Tjaden, J. van der Greef, G. Marko-Varga, L.E. Edholm, J. Chromatogr. A 762 (1997) 193.
[10] Z. Yu, D. Westerlund, Chromatographia 44 (1997) 589.
[11] W.B. Dandliker, V.A. de Sausure, in: M.L. Hair (Ed.), The Chemistry of Biosurfaces, Marcel Dekker, New York, 1971, pp. 1–43.
[12] R.A. Felder, in: G.J. Kost (Ed.), Handbook of Clinical Automation, Robotics and Optimization, Wiley, New York, 1996, Ch. 1.
[13] K. Fredriksson, Ergonomics 38 (1995) 1067.
[14] R.D. McDowall, J.C. Pearce, G.S. Murkitt, Trends Anal. Chem. 8 (1989) 134.
[15] Guide to SPE Automation, Gilson, Villiers-le-Bel, 1997.
[16] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacoby, T. Layloff, C.T. Visvanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.
[17] M.E. Swartz, I.S. Krull, Analytical Method Development and Validation, Marcel Dekker, New York, 1997.