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# AUTOMATED SAMPLE PREPARATION ON-LINE WITH THERMOSPRAY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY FOR THE DETERMINATION OF DRUGS IN PLASMA

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## SUMMARY

The combination of a solid-phase extraction module, the AASP, on-line with thermospray high-performance liquid chromatography–mass spectrometry for the automated determination of drugs in plasma is described. The technique was evaluated successfully using, as an example, the determination of labetalol in human plasma. [$^2H_7$]Labetalol was used as an internal standard to compensate for changes in ionization efficiencies between analyses. The chromatographic and mass spectrometric conditions were optimized for labetalol. The combined technique was demonstrated as being robust and reliable for the analysis of plasma samples from a clinical study.

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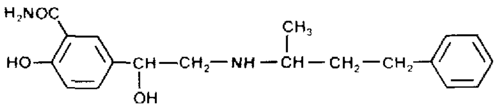
## INTRODUCTION

High-performance liquid chromatography (HPLC) is used extensively for the determination of drugs in biological samples. To handle the large numbers of samples generated during clinical studies, developments have been made in combining sample extraction on-line with HPLC analysis. One of the approaches to this is a semi-automated solid-phase extraction module, the Advanced Automated Sample Processor (AASP)[1].

The mass spectrometer is rapidly fulfilling a role as a selective and universal detector for HPLC. The introduction of the thermospray (TSP) interface[2,3] has provided a robust technique that can be used for the determination of drugs in plasma.



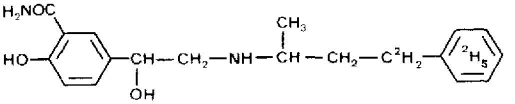
(1)

The combination of an AASP with HPLC–mass spectrometry (HPLC–MS) using a TSP interface is discussed. Labetalol (1), an α- and β-adrenergic receptor antagonist that is effective in the treatment of hypertension in man[4], was selected to evaluate this technique for quantitative analyses. An assay sensitive to 2 ng/ml in plasma using an AASP combined with HPLC and fluorescence detection has been developed for this compound[5]. Therefore, by selecting labetalol as a model for the evaluation, an assessment could be made of the critical analytical and instrumental parameters for the AASP combined with TSP HPLC–MS. This would provide basic information important for the development of future quantitative analyses using the technique.

## EXPERIMENTAL

### *Reagents and materials*

All reagents were of analytical-reagent grade. Labetalol hydrochloride was supplied by Glaxo Central Services Department (Barnard Castle, U.K.). [$^2H_7$]Labetalol (2) was synthesized by the Chemistry Division, Glaxo Group Research (Ware, U.K.). It contained 46% [$^2H_7$]labetalol, 24% [$^2H_6$]labetalol and less than 1% [$^2H_0$]labetalol.



(2)

### *Chromatography*

A Hewlett-Packard 1090 liquid chromatograph was equipped with a binary pumping system. An AASP (Varian, Walton-on-Thames, U.K.) was connected through a Valco ten-port switching valve to the analytical column. Two chromatography systems were used. In system A, a 100 mm × 4.6 mm I.D. stainless-steel column packed with 5 μm Spherisorb CN particles was eluted with methanol–0.1 *M* ammonium acetate (60:40) containing 1% formic acid at a flow-rate of 1 ml/min. In system B, used for the analysis of plasma extracts, a 50 mm × 4.6 mm I.D. stainless-steel column packed with 3 μm Spherisorb ODS-2 particles and protected by a Waters CN Guard-Pak pre-column module (Waters Assoc., Norwich, Cheshire, U.K.) was eluted with methanol–0.1 *M* ammonium acetate (60:40) containing 1% formic acid at a flow-rate of 1 ml/min.

### *Mass spectrometry*

A Hewlett-Packard 5987A mass spectrometer was fitted with a TSP interface and ion source (Vestec, Houston, TX, U.S.A.) and a short-fingered cryopump cooled with liquid nitrogen. The cryopump is not considered essential but it facilitates the continuous use of the system for periods of 8 h or longer. The source and analyser diffusion pumps were backed by an Edwards E2M18 rotary pump (Edwards High Vacuum, Crawley, U.K.). The outlet pump on the TSP ion source was an Edwards E2M8. A Rheodyne filter unit (0.5 μm) was placed in-line between the column outlet and the TSP interface. The ion source tuning parameters were optimized using a solution of labetalol prepared in the eluent for system A. This was pumped directly into the TSP interface at a flow-rate equivalent to 100 ng/s of labetalol. The operating parameters of the interface and ion source were interface tip temperature *ca.* 155°C and ion source 200°C. These were optimized by repetitive analyses of aliquots (5 $\mu$l) of an aqueous solution of labetalol (0.2 mg/ml) injected directly into the TSP interface using the eluent for system A.

Data were acquired and processed using the standard software supplied by Hewlett-Packard. Scanning data were acquired between masses 120 and 400 to avoid the high background resulting from solvent cluster ions at lower mass. Selected ion recording (SIR) data were obtained by monitoring the protonated molecular ions of *m/z* 329 and 336 derived from labetalol and [$^2H_7$]labetalol, respectively. A 350 ms dwell time was used for each ion.

### *Quantitative analysis*

*Preparation of standards.* Calibration standards covering the range 10–103 ng/ml of labetalol in plasma were prepared in control human plasma. Standards of concentration 14 and 48 ng/ml were prepared in control human plasma for replicate analyses to determine the intra-assay variation. The inter-assay variation was determined by triplicate analyses of standards of concentration 15 and 38 ng/ml in plasma on two separate occasions. Any standards not analysed immediately after preparation were stored frozen at −20°C.

*Extraction and analyses.* AASP C-2 cartridges (Jones Chromatography, Llanbradach, U.K.) were conditioned using a Vac-Elut module operated at *ca.* 4 bar air pressure with methanol (1.8 ml) followed by distilled water (1.8 ml). Aliquots (1 ml) of standards or plasma samples received from a clinical study were pipetted into 2 ml vials, [$^2H_7$]labetalol (100 $\mu$l = 114 ng) was added to each and the contents were vortex mixed. These were eluted through the cartridges on the Vac-Elut module. Each cartridge was washed with distilled water (1.8 ml) and the cassettes were loaded on to the AASP module for TSP HPLC–MS analysis using system B. The AASP was operated with a run time of 3 min. The intensities of the ions of *m/z* 329 and 336 were recorded. The SIR chromatograms were smoothed prior to integration.

The ratio of the peak heights of the ions of *m/z* 329 and 336 for each calibration standards was plotted against the concentration of labetalol and a calibration line was determined by a least-squares linear regression analysis. Concentrations of labetalol were determined from the calibration line.

## RESULTS AND DISCUSSION

### *Determination of labetalol and optimisation of instrumental parameters*

Although chromatographic systems had already been developed for the determination of labetalol in human plasma[5–9], these were not compatible with TSP HPLC–MS and a new system (chromatographic system A) was developed.

An ion source temperature of 200°C was the maximum that could be used without the occurrence of major fragmentation. The mass spectrum of labetalol obtained at this temperature showed the protonated molecular ion, *m/z* 329, as the base peak (Fig. 1). The sensitivity was not improved either by using the filament to pro-

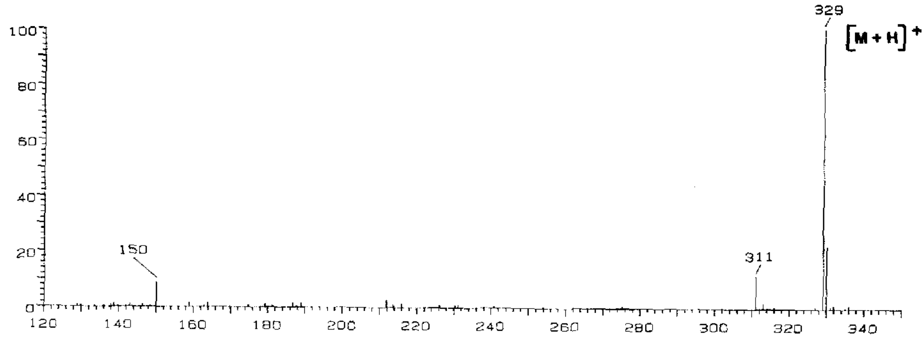


Fig. 1. Mass spectrum of 1 μg of labetalol injected directly into the TSP interface in an eluent of composition methanol–0.1 *M* ammonium acetate containing 1% formic acid (60:40). Source temperature, 200°C.

mote chemical ionization or by operating the mass spectrometer in the negative ion mode. A consequence of the use of the lower source temperature is shown by the mass chromatograms of *m*/*z* 329 in Fig. 2a and b. These compare the peak profiles, obtained at two different source temperatures, for the analysis of 1 μg samples of labetalol injected directly into the eluent. The difference in the peak widths at the two temperatures is probably caused by differences in the desolvation rates of the droplets

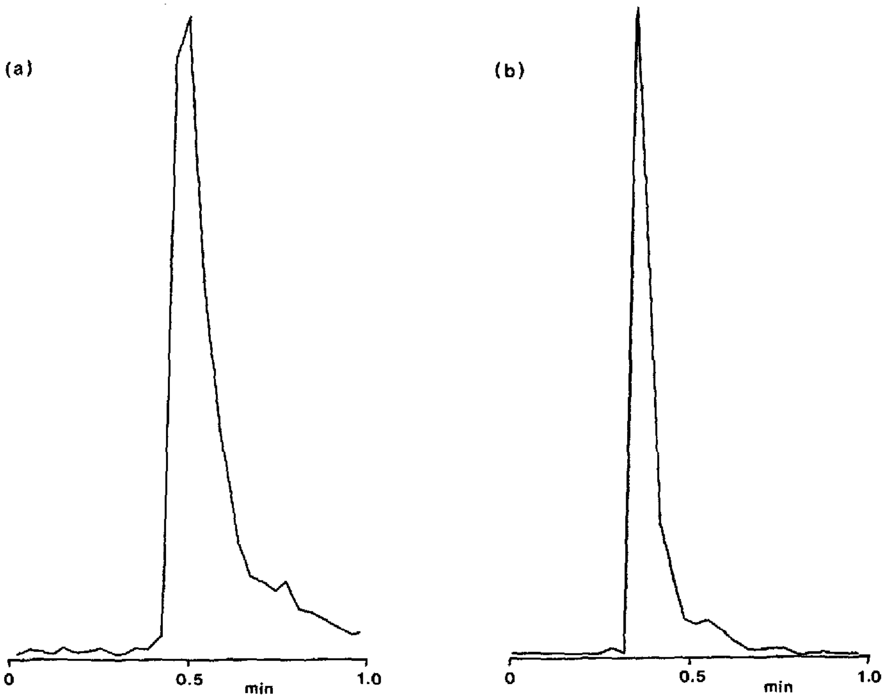


Fig. 2. Mass chromatograms of *m*/*z* 329 derived from 1 μg of labetalol injected directly into the TSP interface in an eluent of composition methanol–0.1 *M* ammonium acetate containing 1% formic acid. Source temperature: (a) 200°C; (b) 300°C.

and different rates in desorption of labetalol that had deposited on the ion source walls. At a source temperature of 300°C the extent of fragmentation varied considerably from injection to injection, making it less suitable for quantitative analysis. It was also observed that at both source temperatures, mass chromatograms of the ions *m/z* 329, $[M + H]^+$, and *m/z* 311, $[M + H - H_2O]^+$, did not coincide. It is likely that this resulted from thermal degradation of labetalol adsorbed on the ion source walls. The effect has been seen for ranitidine using the direct liquid introduction interface (DLI)[10].

Instability in the thermospray jet, which is observed as "spiking" on the peak profile in the reconstructed total ion current chromatogram, has been noted previously[11]. It appeared to be related to the temperature at the tip of the thermospray probe. To reduce "spiking" this temperature was optimized at 5–10°C below the temperature at which complete vaporization of the eluent occurred within the interface. The improvement was critical for subsequent integration of the peaks in the SIR chromatograms.

### *Determination of labetalol in human plasma using an AASP combined with TSP HPLC–MS*

It was necessary to change the HPLC column from nitrile-bonded Spherisorb (system A) to Spherisorb ODS-2 (system B) for the analysis of plasma extracts be-

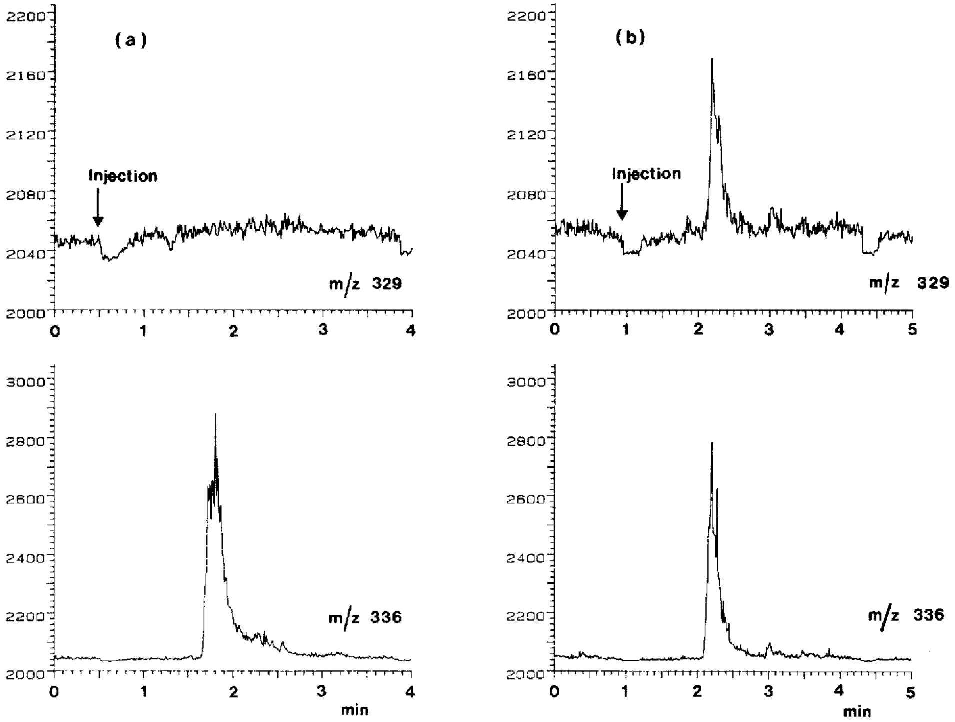


Fig. 3. TSP HPLC–MS analysis of calibration standards using system B. (a) Control plasma containing 114 ng/ml of $[^2H_7]$labetalol; (b) plasma containing 10 ng/ml of labetalol and 114 ng/ml of $[^2H_7]$labetalol. The data were smoothed prior to integration.

TABLE I

DETERMINATION OF THE INTRA-ASSAY VARIATIONS IN THE DETERMINATION OF LABETALOL IN HUMAN PLASMA

| *Nominal concentration (ng/ml)* | *Mean determined (ng/ml)* | *S.D. (ng/ml)* |
|---|---|---|
| 14 | 13.5 ($n$ = 6) | 1.4 |
| 48 | 49.6 ($n$ = 5) | 1.3 |

cause the level of background interference in the $m/z$ 329 channel was unacceptably high when system A was used. Using system B, the SIR chromatograms for two calibration standards, a control and 10 ng/ml of labetalol in plasma, are shown in Fig. 3a and b. Each standard contained 114 ng of [$^2H_7$]labetalol. By combining the AASP extraction module with TSP HPLC–MS, the total extract from 1 ml of plasma was analysed by the mass spectrometer. Fig. 3a shows that there are no interfering ions derived from compounds endogenous to plasma in the $m/z$ 329 channel. A similar profile, not shown here, was obtained in the $m/z$ 336 channel for control plasma analysed in the absence of the internal standard. The low background level resulted in a fast analysis time of approximately 3 min per sample.

The SIR chromatograms derived from $m/z$ 329 show a drop in the baseline level during the injection period. This occurred when the AASP cartridge was placed automatically in-line with the HPLC column. The cartridge contained trapped air and when the eluent was directed through it an initial drop in back-pressure occurred. This caused a reduction in the flow-rate, with the result that complete vaporization of the eluent occurred within the TSP interface. However, as Fig. 3 shows, stable conditions were established rapidly. Although the TSP technique is robust enough to handle this, the jet produced in DLI would have been destroyed.

The assay had a limit of detection of approximately 5 ng/ml. However, after the source had been cleaned by electropolishing, the limit of detection deteriorated; 100 ng of labetalol in an aqueous solution could just be measured. The original limit of detection was achieved only after several analyses of plasma extracts. This suggested irreversible adsorption of plasma constituents on to the ion source, thereby reducing the adsorption of labetalol.

The calibration graph of (peak height at $m/z$ 329)/(peak height at $m/z$ 336) *versus* ng/ml of labetalol in plasma was linear over the range 10–103 ng/ml with $r$

TABLE II

DETERMINATION OF THE INTER-ASSAY VARIATIONS IN THE DETERMINATION OF LABETALOL IN HUMAN PLASMA

| *Nominal concentration (ng/ml)* | *Assay 1* | | *Assay 2* | |
|---|---|---|---|---|
| | *Mean determined (n = 3) (ng/ml)* | *S.D. (ng/ml)* | *Mean determined (n = 3) (ng/ml)* | *S.D. (ng/ml)* |
| 15 | 14.6 | 1.2 | 15.7 | 1.5 |
| 38 | 38.0 | 2.0 | 40.3 | 3.5 |

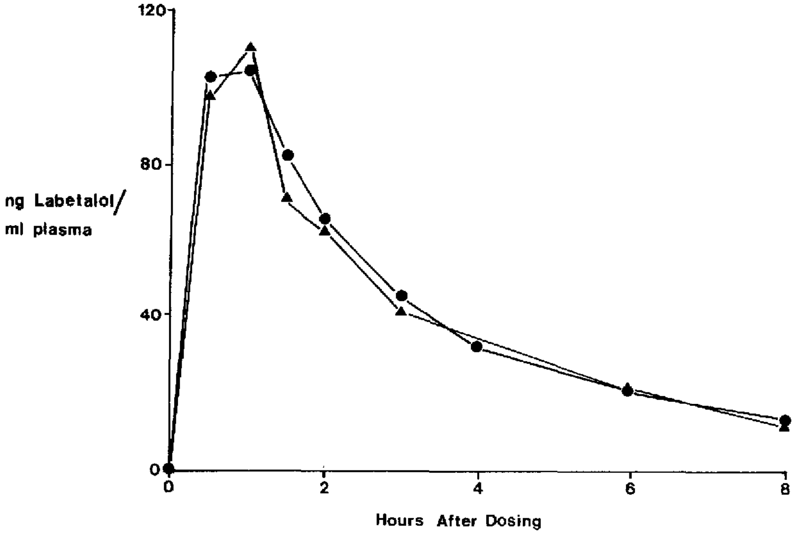


Fig. 4. Comparison of HPLC–MS (▲) with HPLC and fluorescence detection[5] (●) for the analysis of plasma samples obtained following the administration of a 200 mg labetalol tablet to a volunteer.

= 0.9994. An internal standard, labelled with a stable isotope, was necessary to compensate for changes in ionization efficiencies between analyses. This variation in efficiency was measured by determining the coefficient of variation of the peak height of the ion of $m/z$ 336 derived from [$^2H_7$]labetalol in each of the calibration standards. This was found to be 47%, which was greater than could be attributed to analytical errors and could only be due to variations in ionization efficiencies occurring within the ion source. The intra- and inter-assay variabilities determined at two concentrations are given in Table I and II.

Fig. 4 compares the results obtained by TSP HPLC–MS with those obtained by an established HPLC assay[5] for the analysis of the same series of plasma samples. A good correlation was obtained between the two methods.

## CONCLUSION

The combination of a semi-automated sample preparation module, the AASP, with thermospray HPLC–MS was evaluated successfully by the determination of labetalol in human plasma. The combined technique is an extremely useful method for routine handling of biological samples but, although robust, it is not yet at the stage of development where samples can be analysed unattended overnight. Furthermore, careful optimization of the instrumental parameters and the use of a stable isotopically labelled analogue as an internal standard are required for each compound studied.

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