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Journal of Pharmaceutical and Biomedical Analysis
16 (1998) 777–783

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Automated 96-well solid phase extraction for the determination of doramectin in cattle plasma

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Received 6 March 1997; received in revised form 10 April 1997

Abstract

Automated standard and sample preparation have been coupled with 96-well solid phase extraction (SPE) technology to produce a cost effective, high throughput system for the analysis of drugs in biological media. The system was originally designed using the Packard Multiprobe 104DT™ robotic sample processor (RSP) to improve throughput for the assay of doramectin in cattle plasma, and the assay has since been validated (0.5–100 ng ml^{-1}) using the Tecan Genesis RSP 150/8™. The robotic processor conducts all liquid handling procedures involved in sample extraction. These comprise preparation of calibration standards in plasma, dispensing and diluting of plasma samples and addition of internal standard. In addition, the robot primes the 96-well SPE block, applies calibration standards and samples, draws the mixtures through the 96-well SPE block, and finally washes the block ready for manual elution. The doramectin assay involves high-performance liquid chromatography (HPLC) with fluorescence detection, and requires the sample extracts to be derivatised prior to analysis. The derivatisation procedure is performed manually in situ in the polypropylene deep 96-well block into which the samples have been eluted from the SPE-block. The derivatised samples are taken directly from the deep well block and injected into the HPLC for analysis. This type of batch processing keeps sample transfer to a minimum. Automated sample preparation, in combination with the use of 96-well technology, has reduced both cost and effort required in the analysis of doramectin in cattle plasma samples, and has resulted in improved sample throughput. © 1998 Elsevier Science B.V.

Keywords: Automated sample preparation; Doramectin; Laboratory robotics; 96-Well solid phase extraction block

1. Introduction

Solid phase extraction (SPE) is a valuable and widely used technique for the separation of analytes (drugs) from their surrounding matrix (e.g., plasma). Conventionally, SPE is carried out using individual cartridges packed with the sorbent of choice. The 96-well solid phase extraction block is an array of SPE cartridges in a 12 × 8 format [1]. The block is set to revolutionise plasma analysis following administration of drugs to animals and humans. The advantages of batch processing over single cartridges include time saving and cost effectiveness. Despite these savings, considerable time and effort are still required to wash and prime the block, and to draw samples through the

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PII S0731-7085(97)00110-6

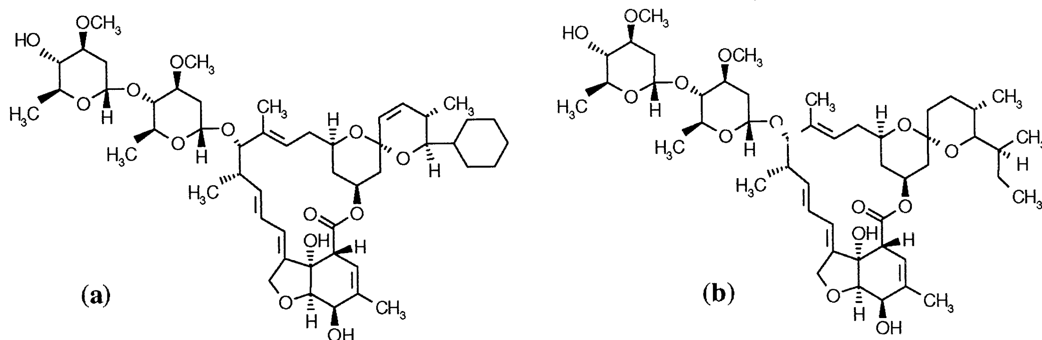

Fig. 1. Structures of (a) doramectin and (b) internal standard (22,23-dihydroavermectin $B1_a$).

solid phase medium, using a vacuum. This type of assay lends itself to automation, and the validation of such an assay is described herein.

Doramectin (25-cyclohexyl-5-*O*-demethyl-25-de(1-methylpropyl)avermectin $A1_a$; see Fig. 1(a)) is a fermentation-derived avermectin with broad spectrum antiparasitic activity in cattle [2]. Doramectin is currently marketed (by Pfizer) as a livestock antiparasitic agent (Dectomax™).

Studies with the first commercialised avermectin, ivermectin [3,4], and subsequently doramectin [5], have shown that pharmacokinetics and efficacy can be affected by rate of absorption from the subcutaneous administration site. In order to monitor doramectin pharmacokinetics in large-scale cattle studies, a sensitive and high throughput quantitative assay was required for the determination of doramectin in cattle plasma. The assay itself is complex and time consuming, involving sample dilution, solid phase extraction, derivatisation and HPLC analysis. The whole process has been simplified using 96-well SPE technology, with automated sample preparation. The automated system was developed in conjunction with Packard, using the Multiprobe 104DT™ robotic sample processor, and the methodology has since been transferred to and validated using the Tecan Genesis RSP 150/8™. Both systems required minor customisation to allow the robot to control a vacuum switching valve, and allowed unattended sample preparation and solid phase extraction.

2. Experimental

2.1. Materials

Super-purity-grade methanol and acetonitrile were obtained from Romil (Cambridge, UK). Tetrahydrofuran (SLR grade) was obtained from Fisher Scientific (Loughborough, UK). Triethylamine, trifluoroacetic anhydride and 2.0 M ammonia solution in methanol were obtained from Aldrich (Gillingham, Dorset, UK). The 96-well solid phase extraction blocks (Microlute™), fitted with extension reservoirs and packed with 50 mg IST-C_{18} sorbent per well, and vacuum manifolds, were obtained from Porvair Sciences (Shepperton, UK). Polypropylene deep well plates were obtained from Barden Engineering (Whitstable, Kent, UK). Polypropylene flat-bottomed tubes were obtained from Sarstedt (Leicester, UK). Tomtech Thinlids were obtained from Receptor Technologies (VT, USA).

2.2. Instrumentation and chromatography conditions

Chromatographic separations were performed using a Spherisorb S5C8 column, 25 cm × 4.6 mm i.d. (Hichrom, Reading, UK), and using a mobile phase consisting of 67.5% acetonitrile, 17.5% water and 15% tetrahydrofuran, with a flow rate of 1 ml min^{-1}. The eluent was monitored with a LaChrom L-7480 fluorescence detector, fitted with a 12 μl flow cell (BDH-Merck, Poole, UK), and

Table 1
Calibration curve reproducibility; individual and mean data from three separate runs

Concentration (ng ml^{-1})					S.D.	Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean			
0.5	0.57	0.47	0.45	0.50	0.06	−0.7	13
1	1.0	1.0	1.0	1.0	0.0	0.0	0.0
2	2.0	2.1	2.1	2.1	0.1	3.3	2.8
5	4.9	5.1	5.1	5.0	0.1	0.7	2.3
10	9.8	10	10	10	0.3	0.7	2.5
20	19	19	19	19	0.3	−5.7	1.3
50	48	47	48	48	0.4	−4.6	0.8
100	104	103	104	104	0.3	3.5	0.2
r^2 (1/*y*)	0.9981	0.9979	0.9974	0.9978	0.0004	—	0.04
					Mean	−0.4	2.9

[a] Accuracy = [(mean measured value − prepared value)/(prepared value)] × 100.
[b] Precision = (S.D. × 100)/mean.

set at an excitation wavelength of 360 nm and an emission wavelength of 470 nm. A Shimadzu LC-9A HPLC pump (Dyson Instruments, Houghton-Le-Spring, UK) and Merck-Hitachi AS-4000 autosampler, with a 200 µl injection loop (BDH-Merck, Poole, UK), were used. A Techne sample concentrator (Techne, Loughborough, UK), adapted to hold 96 tips, was used for solvent evaporation following solid phase extraction.

Automated sample preparation and solid phase extraction were carried out using a Tecan Genesis RSP 150/8™ (using disposable tips) which had been specially adapted (by Tecan) to control a vacuum switching valve (Tecan UK, Goring-on-Thames, UK). A custom-written programme controls the switching of the valve via a relay adaptor card fitted to the computer (Labstar Software, Strathaven, Lanarkshire, UK). The in-house vacuum line (−625 mmHg) was used for drawing liquids through the SPE block. A Proline 8-channel dispenser (Alpha Laboratories, Eastleigh, Hampshire, UK) was used for dispensing methanol for sample elution, and a Multichannel Microdispenser Model 868 (Camlab, Cambridge, UK) was used for dispensing derivatisation reagents, following solid phase extraction.

2.3. Preparation of solutions

A stock solution of doramectin (200 µg ml^{-1}) was prepared in methanol in a glass volumetric flask and stored at 4°C. A working solution (50 µg ml^{-1}) was prepared by dilution of the stock with methanol and stored at 4°C. The working solution was added to 4 ml of control calf plasma to give a final concentration of 100 ng ml^{-1}. This solution was freshly prepared for each run and was used in the preparation of the standard curve. The quality control (QC) samples were prepared in three pools of 50 ml, to give final concentrations of 0.5, 50 and 100 ng ml^{-1}, and were dispensed ($n = 7$) into flat-bottomed, screw-capped polypropylene tubes, and stored at −20°C. A stock solution of internal standard (22,23-dihydroavermectin $B1_a$ (DHAVMB1_a; Fig. 1(b)), 30 µg ml^{-1}) was prepared in methanol, in a glass volumetric flask, and stored at 4°C. An internal standard working solution was freshly prepared for each run by adding the stock to an appropriate volume of 30% acetonitrile in water, to give a final concentration of 20 ng ml^{-1}. All plasma samples were centrifuged (2500 × *g*, 10 min) prior to analysis. Stock solutions were prepared monthly and working solutions were freshly prepared as required.

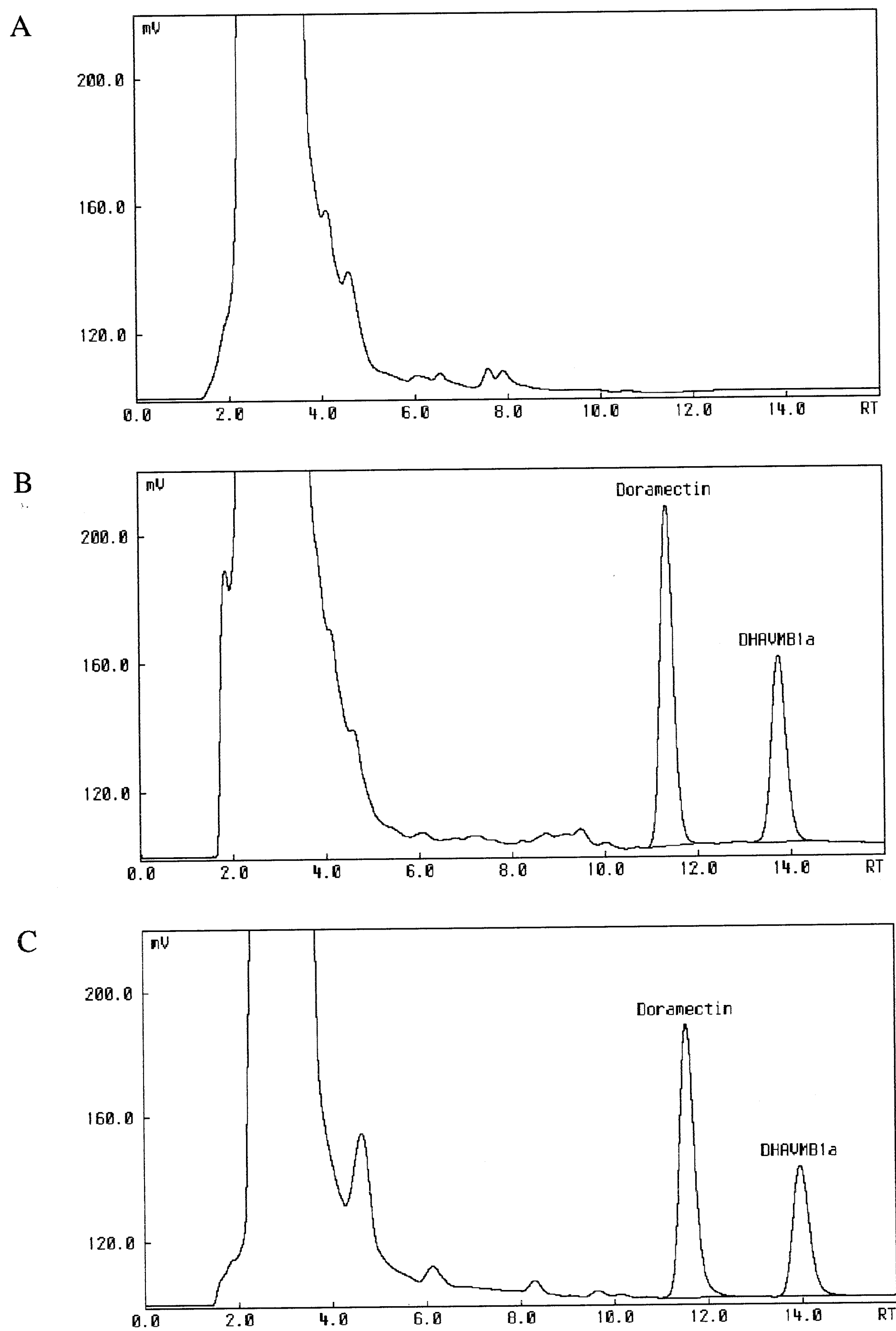

Fig. 2. Representative chromatograms of doramectin and internal standard ($DHAVMB1_a$) in cattle plasma (1 ml). (A) Control plasma blank; (B) control plasma spiked with 20 ng ml^{-1} doramectin and 10 ng ml^{-1} $DHAVMB1_a$; (C) 24 h plasma sample from calf administered doramectin (500 μg kg^{-1} s.c.) spiked with 10 ng ml^{-1} $DHAVMB1_a$.

Table 2
Intra-day accuracy and precision for the analysis of doramectin in cattle plasma

Concentration (ng ml^{-1})		S.D.	Accuracy (%)	Precision (%)	n
Prepared	Mean measured				
0.5	0.57	0.03	14.3	4.5	7
50	53	3.7	6.9	7.0	7
100	107	2.1	7.2	2.0	7

2.4. Robot assay

Automated steps carried out by the Tecan Genesis were as follows.

2.4.1. Preparation of 96-well SPE block

The extraction block (situated on the vacuum manifold) was primed by the addition of 1 ml of methanol to each channel to be used (unused channels were sealed off using a self-adhesive acetate sheet). The methanol was drawn through the sorbent at a rate of 100 μl min^{-1}, by applying a pulsed vacuum to the manifold (75 ms on, 5000 ms off, total cycle time 10 min). The block was then washed by adding 1 ml water to each channel and drawing through at a rate of 3 ml min^{-1} under vacuum (vacuum switched on for 20 s).

2.4.2. Preparation of standards

Standards at final concentrations of 0, 0, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng ml^{-1} were prepared by diluting the 100 ng ml^{-1} working plasma solution with control calf plasma, in flat-bottomed polypropylene tubes, to give a final volume of 1 ml. Samples were mixed by aspirating and dispensing.

2.4.3. Dispensing of QC samples

Aliquots (1 ml) of each QC sample ($n = 7$ at each concentration) were transferred into flat-bottomed polypropylene tubes. Internal standard solution (0.5 ml) was added to each standard and QC, with the exception of the first (blank) standard, which received 0.5 ml of 30% acetonitrile in water. Following addition of internal standard solution (or 30% acetonitrile), samples were mixed by repeat ($n = 4$) aspirating and dispensing.

2.4.4. Solid phase extraction

Directly after mixing, the total volume of each sample was transferred onto the 96-well SPE block. Once the transfer was complete, samples were drawn through the block at a rate of 75 μl min^{-1}, by applying a pulsed vacuum (100 ms on, 5000 ms off, total cycle time 20 min). Water (1 ml) was then added to each well to wash the sorbent. The water was drawn through the sorbent at a rate of 3 ml min^{-1}, by applying a vacuum to the manifold (vacuum on for 5 min).

2.5. Manual steps

The following steps of the assay were carried out manually.

2.5.1. Sample elution

The Microsep block was transferred from the waste manifold onto a 96-well deep polypropylene plate which was situated in the elution manifold. Methanol (1 ml) was then added to each channel, using the Proline 8-channel dispenser. The methanol was drawn through the sorbent (100 μl min^{-1}) under vacuum, and captured in the corresponding wells of the deep well plate. The methanol was then evaporated to dryness (60°C under air) in situ using the 96-place Techne sample concentrator (tips were cleaned prior to use by immersing in methanol and then allowing to dry).

2.5.2. Sample derivatisation

Samples were derivatised to give a fluorescent product, using the method of Nowakowski et al. [6]. Triethylamine (100 μl of 50% v/v in acetonitrile) was added to each well, using the Multichannel Microdispenser, and the plate was gently

Table 3
Inter-day accuracy and precision for the analysis of doramectin in cattle plasma (calculated from mean data from three separate runs)

Concentration (ng ml^{-1})		S.D.	Accuracy (%)	Precision (%)	n
Prepared	Mean measured				
0.5	0.57	0.06	13.3	1	3
50	53	1.6	6.5	3	3
100	108	1.4	8.4	1.3	3

shaken. Trifluoroacetic anhydride (150 µl of 33% v/v in acetonitrile) was then added to each well, producing an intense yellow-coloured solution. The plate was again gently shaken, and samples concentrated to approximately 100 µl in the sample concentrator. Ammonia solution (250 µl of 2.0 M in methanol) was then added to each well, the plate gently shaken, and the samples concentrated to approximately 100 µl in the sample concentrator. Acetonitrile (100 µl) was then added to each well and the plate gently shaken. The plate was then sealed with a Tomtech Thinlid, and placed in the autosampler for direct sampling (sample volume 100 µl) for HPLC analysis.

2.5.3. Data acquisition and analysis

Peak heights for doramectin and $DHAVMB1_a$ were acquired and processed using Multichrom Version 2.1 (Labsystems, Manchester, UK). Calibration lines were constructed by plotting peak height ratios of drug to internal standard against drug concentration. A weighted ($1/y$) linear regression line was fitted, and drug concentrations in the standards and QC samples were interpolated from this line. The weighting was used due to the large range (200-fold) of the calibration standard concentrations.

3. Results and discussion

3.1. Calibration curves

Calibration curves were shown to be linear from 0.5 to 100 ng ml^{-1}. Back calculated linear regression calibration data are shown in Table 1. A mean correlation coefficient (r^2) of 0.9978 was obtained from the three runs, prepared on separate days. The calibration data were associated with a mean precision value of -0.4% and mean accuracy value of 2.9%, showing good between run reproducibility in constructing calibration standards. All standards were prepared by the robot to within 6% of their nominal concentrations, with precision values within 3% for all except the 0.5 ng ml^{-1} standard (13%).

3.2. Assay validation

Typical chromatograms are shown for control plasma and for plasma spiked with doramectin (20 ng ml^{-1}) and internal standard (Fig. 2). Doramectin and internal standard were eluted with retention times of 11.3 and 13.7 min, respectively. Fluorescence detection combines selectivity, as can be seen by comparing blank and spiked plasma chromatograms (Fig. 2), with sensitivity (lower limit of quantification of 0.5 ng ml^{-1}). Extraction efficiency was 73% for doramectin and 49% for internal standard.

Accuracy and precision of the assay were determined by analysing the QC samples (prepared from an independent solution to the calibration standards) on three separate occasions. The intraday accuracy (<14.3%) and precision (<7.0%) for this assay were considered satisfactory [7] (Table 2), with a precision of 4.5% on replicates at 0.5 ng ml^{-1}; this was defined as the lower limit of quantification for doramectin in cattle plasma. Furthermore, inter-day accuracy (<13.3%) and precision (<3%) demonstrated the good reproducibility of the assay (Table 3). The lower accuracy of the 0.5 ng ml^{-1} QC, compared to those of the 50 and 100 ng ml^{-1} QC samples (Tables 2

and 3), is due to the low signal-to-noise ratio in the analysis of the lowest concentration QC.

The reproducibility and robustness of this assay allow high sample throughput, with minimal manual input. A maximum of 96 samples (including standards) can be processed and extracted by the robot in under 90 min, freeing the user for other tasks. The time taken for the manual stages (approximately 2 h, including sample evaporation) is independent of sample number, since samples are contained in the deep well block and are manipulated simultaneously. The advantage of in situ derivatisation and direct sampling from the deep well block is that no further sample transfer is required once samples have been dispensed into the extraction block by the robot. This minimises errors brought about by repetitive manual procedures. The rate-limiting step for this assay is HPLC analysis, which takes 24 h for 96 samples. However, this step can be condensed, if required, by using more than one analysis system, or by modification of HPLC conditions to shorten run times. A combination of automation and 96-well technology has resulted in an increase in daily throughput of at least 20% for the assay of doramectin in cattle plasma. In addition to improving throughput and freeing time for the user, the 96-well SPE assay is cheaper to run when counting the cost of consumables; cost per sample (calculated for 96 samples) is 45% of that when analysing by conventional SPE.

4. Conclusion

Employing the Microsep 96-well solid phase extraction block for the assay of doramectin in cattle plasma has enabled the development of a semi-automated assay, using a robotic sample processor. The logical coupling of these two technologies has potentially provided the means for high throughput, fully automated sample preparation for the analysis of drugs in biological media. The availability of a wide range of sorbents in the Microsep block format means that most SPE applications can be converted to run on a system such as the one described here. Indeed, other avermectins (ivermectin and moxidectin), and some Pfizer human medicinal discovery compounds, have also been extracted using the above described system. The system has also been used in the development of discovery formulations for drug delivery. In addition to flexibility and increased sample throughput, the savings in cost of consumables and operator time further add to the justification of this technique.

Acknowledgements

The authors would like to thank Steve Jope of Packard for his input in developing and customising the Packard Multiprobe 104DT™ to enable 96-well SPE operation. Also, thanks go to Frank Smith and Sarah Ireland (Tecan UK) for their assistance in transferring the methodology to the Tecan Genesis RSP 150/8™, and to Ken Saunders (Drug Metabolism, Pfizer Central Research, Sandwich) for his interest and advice whilst setting up this automated assay.

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