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Rapid determination of the anti-cancer drug chlorambucil (Leukeran™) and its phenyl acetic acid mustard metabolite in human serum and plasma by automated solid-phase extraction and liquid chromatography–tandem mass spectrometry

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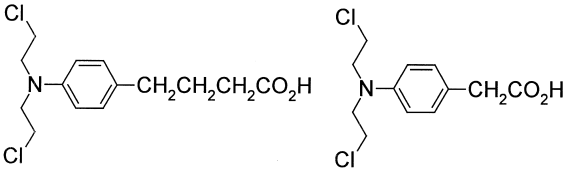
Abstract

A bioanalytical method for the determination of the anticancer drug chlorambucil (Leukeran™) and its phenyl acetic acid mustard metabolite in human serum and plasma is described. Automated solid-phase extraction of the analytes is carried out with C_{18} sorbent packed in a 96 well format microtitre plate using a robotic sample processor. The extracts are analysed by isocratic reversed-phase liquid chromatography using pneumatically and thermally assisted electrospray ionisation (TurboIonspray) with selected reaction monitoring. The method is specific and sensitive, with a range of 4–800 ng/ml in human serum and plasma for both parent drug and metabolite (sample volume 200 μl). The method is accurate and precise with intra-assay and inter-assay precision (C.V.) of <15% and bias <15% for both analytes. The automated extraction procedure is significantly faster than manual sample pre-treatment methods, a batch of 96 samples is extracted in 50 min allowing for faster sample turnaround. The method has been used to provide pharmacokinetic support to biocomparability studies of Leukeran™ following single doses of oral tablet formulations.

Keywords: Chlorambucil; Leukeran; Phenyl acetic acid mustard

1. Introduction

Chlorambucil (Leukeran™, Fig. 1) is a bifunctional alkylating agent used in the treatment of Hodgkin's disease, malignant lymphomas, chronic lymphocytic leukaemia and ovarian and breast carcinomas [1,2]. Chlorambucil is extensively metabolised at the butyric acid side chain by β-oxidation, yielding the cytotoxic metabolite phenyl acetic acid mustard (PAAM) (Fig. 1) which itself exhibits anti-



Chlorambucil phenyl acetic acid mustard

Fig. 1. Structures of chlorambucil and phenyl acetic acid mustard (PAAM).

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cancer activity [3,4]. Studies have shown that following oral administration of chlorambucil, metabolism to PAAM is rapid and exposure to the metabolite is greater than to that of the parent compound [5,6]. Information on in vivo metabolite concentrations is therefore important when patients undergo treatment with chlorambucil.

In order to support a clinical study to examine the biocomparability of three formulations of chlorambucil in human patients with refractory malignancies, a liquid chromatography–tandem mass spectrometry (LC–MS–MS) based bioanalytical method was developed and validated for chlorambucil and PAAM in human serum and plasma. A robust method, able to rapidly turnaround samples, was required with a lower limit of quantitation of 4 ng/ml.

A number of analytical methods for the determination of chlorambucil, its metabolites and derivatives have been reported in the scientific literature [7–16]. These methods were not considered suitable for our requirements however. The method of Leff et al. [7] involved time consuming liquid–liquid extraction and derivatisation and the limit of detection was 5 μg/ml. The method of Newell et al. [8] involved a 10 min chromatographic run time and did not offer the required sensitivity. Chang et al. [9] reported a GC–MS method, however, the sample preparation involved liquid–liquid extraction followed by derivatisation and gave a detection limit of 50 ng/ml. The chromatographic system reported by Chatterji et al. [10] produced a run time of 10 min with a quantifiable range of 10–200 μg/ml. The HPLC–UV method of Ahmed et al. [11] produced a retention time of 8 min for chlorambucil following extraction from plasma with acetonitrile and provided a lower limit of detection of 0.1 μg/ml. Adair et al. [12,13] compared the responses given by UV, fluorescence and electrochemical detection and found UV detection to give the optimum response. The limit of detection achieved was 10 ng/ml although this was obtained from a sample volume of 3 ml. The extraction procedure was lengthy, employing protein precipitation and extraction cartridge techniques. The HPLC–UV methods reported by Oppitz et al. [14] and Greig et al. [15] involved long chromatographic run times (12–24 min) and used reagents unsuitable for mass spectrometric detection. These methods did not offer the required sensitivity with lower limits of quantification of 30 ng/ml and 100 ng/ml respectively. The method reported by Chandler et al. [16] involved solvent extraction and the chromatographic run time was 30 min.

These older methods involve lengthy sample pretreatment, sample preparation and/or chromatographic run times and in some cases use considerable volumes of solvent and reagent. They were not considered suitable for our purposes since fast sample turnaround was required. Also the lower limits of quantification were not adequate for the clinical trial support planned. This report describes the use of rapid automated 96 well solid-phase extraction (SPE) [17] using sample and solvent volumes of ≤200 μl. The use of MS–MS provides greater specificity than UV detection. Improved limits of detection would be desirable when pharmacokinetic measurements are required after low doses and to limit the percentage of the AUC extrapolated especially when testing for bioequivalence between different formulations.

2. Experimental

2.1. Chemicals and reagents

Chlorambucil was obtained from Sigma Chemical Company (Poole, UK), and phenyl acetic acid mustard (PAAM), [$^{13}C_4^2H_4$]-chlorambucil and [$^{13}C_4^2H_6$]-PAAM were synthesised within Glaxo Wellcome Laboratories, Stevenage, UK, and Research Triangle Park, US. HPLC grade acetonitrile was obtained from Romil Chemicals (Cambridge, UK), and Analar grade formic acid and hydrochloric acid were obtained from BDH (Poole, UK).

2.2. Preparation of stock solutions, calibration standards and quality control samples

Nitrogen mustard compounds degrade in aqueous media to their mono and dihydroxy derivatives at room temperature [6]. For stability purposes blood samples were taken into lithium heparin tubes stored in ice prior to separation of the plasma.

Duplicate analytical stock solutions of chlorambucil and PAAM were prepared by dissolving the

reference materials in acetonitrile with 1% (v/v) 8 *M* HCl for stability purposes [10,18], obtaining 500 μg/ml concentrations. Mixed analyte solutions at appropriate concentrations were prepared from these in the acidified acetonitrile. Heparinised control human plasma, obtained from normal human volunteers, was spiked with appropriate volumes of the mixed analyte solutions to give calibration standards at 4, 10, 50, 100, 300, 500 and 800 ng/ml for both chlorambucil and PAAM. Quality control samples were similarly prepared at 10, 200 and 600 ng/ml for both analytes using a separate set of solutions.

Solutions of [$^{13}C_4^2H_4$]-chlorambucil and [$^{13}C_4^2H_6$]-PAAM for use as internal standards were prepared in the same way as the analytical stock solutions at concentrations of 250 μg/ml. Aliquots of the internal standards were combined and diluted with 0.1% (v/v) formic acid on the day of an analytical run.

2.3. Solid-phase extraction

All plasma samples were thawed and mixed thoroughly on a reciprocal mixer for 10 min and then centrifuged (1800 *g* for 10 min) prior to extraction. Samples were extracted using 96 well MicroLute™ solid-phase extraction (SPE) blocks (Porvair Sciences Ltd., Shepperton, Middlesex, UK) packed with 15 mg of C_{18} sorbent per well. The MicroLute™ blocks were extracted using a MultiPROBE 104DT robotic sample processor (RSP, Canberra-Packard, Pangbourne, Berks., UK) and a Porvair acrylic vacuum manifold (Porvair Sciences Ltd., Shepperton, Middlesex, UK). The robotic sample processor (RSP) was programmed to condition each well sequentially with 150 μl aliquots of acetonitrile followed by 0.1% (v/v) formic acid. After conditioning the block, the RSP loaded 200 μl of sample together with 150 μl of 0.1% (v/v) formic acid containing both isotopically labelled internal standards at approximately 0.2 μg/ml. The 96 well block was washed sequentially with further 150 μl aliquots of 0.1% (v/v) formic acid. A deep well microtitre plate was then placed within the vacuum manifold in order to collect the eluent. The compounds were eluted from the sorbent with 100 μl of mobile phase. The plate was sealed with aluminium foil and transferred to the autosampler for analysis by LC–MS–MS. The SPE procedure is detailed in Table 1.

3. Liquid chromatography–tandem mass spectrometry (LC–MS–MS)

The HPLC system comprised an autosampler 233 XL (Gilson, Anachem, Luton, UK), a PU-980 pump, LG-980-02 ternary gradient unit, DG-980-50 degasser and CO-960 column oven (Jasco, Great Dunmow, Essex, UK). Detection was by means of an API-III$^+$ triple quadrupole mass spectrometer (PE-Sciex, Ontario, Canada).

The mobile phase consisted of 0.1% (v/v) formic acid and 0.1% (v/v) formic acid in acetonitrile mixed in the ratio 30:70 (v/v) and pumped at a flow-rate of 1 ml/min. Analytes were separated on a 10×4.6 mm Supelcosil LC-18 column, 5 μm particle size (Sigma Chemical Company, Poole, UK), maintained at 40°C. The injection volume was 20 μl and the needle wash consisted of 60% (v/v) aqueous acetonitrile in order to eliminate carryover.

The column effluent was split with 900 μl/min directed to waste and only 100 μl/min entering the TurboIonspray interface operated in positive ion

Table 1
SPE procedure

Step	Process	Volume (ml)	Vacuum (s)
1	Acetonitrile (prime)	0.15	10
2	0.1% (v/v) formic acid (prime)	0.15	10
3	Internal standard sample (load)	0.15 0.20	30
4	0.1% (v/v) formic acid (prime)	0.15	30
5	Mobile phase (elution)	0.10	30

mode. The nebulizer temperature was maintained at 450°C and nitrogen was used as both the nebulizer gas and auxiliary gas with flow-rates of 1.2 l/min and 6 l/min, respectively. Argon was used as the collision gas at an indicated thickness of 290. The analytes were detected by selected reaction monitoring (SRM) of the transitions m/z 304–192 for chlorambucil, m/z 312–172 for [$^{13}C_4^2H_4$]-chlorambucil, m/z 276–164 for PAAM and m/z 286–167 for [$^{13}C_4^2H_6$]-PAAM.

Chromatographic peaks were integrated using the PE-Sciex MacQuan processing software. Calibration curves were constructed by plotting peak area ratios of analyte to internal standard against concentration, using a weighted (1/X) linear regression model. Concentrations of the analytes in unknown, QC and Validation Control (VC) samples were subsequently interpolated from these curves. A one-way analysis of variance (ANOVA) of the validation data was performed by an Excel macro (Microsoft Corp.)

4. Results and discussion

4.1. Mass spectrometry

The full scan, positive ion TurboIonspray mass spectrum for chlorambucil acquired by LC–MS is shown in Fig. 2A. The spectrum is dominated by the protonated molecule at m/z 304, which exhibits a characteristic chlorine isotope pattern and a similar MH^+ ion at m/z 276 was observed in the spectrum of PAAM which is shown in Fig. 2C. Similar spectra are obtained for the internal standards [$^{13}C_4^2H_4$]-chlorambucil and [$^{13}C_4^2H_6$]-PAAM dominated by protonated molecules at m/z 312 and 286 respectively, however the chlorine isotope pattern is complicated by the deuterium distribution. These spectra are presented in Fig. 2B,D, respectively.

The corresponding product ion LC–MS–MS mass spectra for the MH^+ ions of chlorambucil, [$^{13}C_4^2H_4$]-chlorambucil, PAAM and [$^{13}C_4^2H_6$]-PAAM are shown in Fig. 3A–D, respectively. The MH^+ ion of chlorambucil undergoes fragmentation at the butyric acid and chloroethyl side chains to give product ions at m/z 192 and m/z 168. The equivalent product ions are observed in the spectrum of [$^{13}C_4^2H_4$]-chlorambucil at m/z 193 and m/z 172 indicating loss of isotopic label from the chloroethyl side chains. The MH^+ ion of PAAM yields a product ion at m/z 164 and the corresponding product ion of [$^{13}C_4^2H_6$]-PAAM is at m/z 167. For optimum sensitivity the product ion m/z 192 was chosen for the selected reaction monitoring (SRM) transition for chlorambucil, however the product ion at 172 was selected for the internal standard for greater mass resolution and since it was of similar intensity. For PAAM and its internal standard the product ions at m/z 164 and m/z 167 respectively were chosen for selected reaction monitoring.

4.2. Solid-phase extraction and chromatography

Separation of chlorambucil and its metabolite PAAM was achieved in under 2 min and since the mass selective nature of the detection instrument allowed resolution of the isotopically labelled internal standards, chromatographic run times were kept to a minimum. The LC–MS–MS SRM chromatograms of an extracted calibration standard at 4 ng/ml for chlorambucil and PAAM (the lower limit of quantification of the method) are shown in Fig. 4. Sample preparation hence became the rate-limiting step in the method. In previous methods [7–16], sample preparation was by protein precipitation with solvent/acid or solvent extraction, followed by centrifugation and further pre-treatment (sample dry down, filtering, and sample freezing). These techniques were performed manually and would take a number of h. It is unlikely that a batch size as large as 96 samples would have been processed using these manual methods and 96 samples could not be extracted in the 50 min processing time reported in this document. In the method reported here 96 well SPE is employed, which has been shown to significantly reduce sample preparation time [19]. The time saved during sample preparation is likely to be measured in h/day. The 96 well SPE procedure was automated by means of a RSP which performs all the liquid handling steps (see Table 1). Although fixed tip probes were used on the RSP, the tips were extensively washed between aspirations and no carry over was observed. The extraction recovery was estimated by comparing the responses obtained from human serum extracts at concentrations of 50 ng/ml,

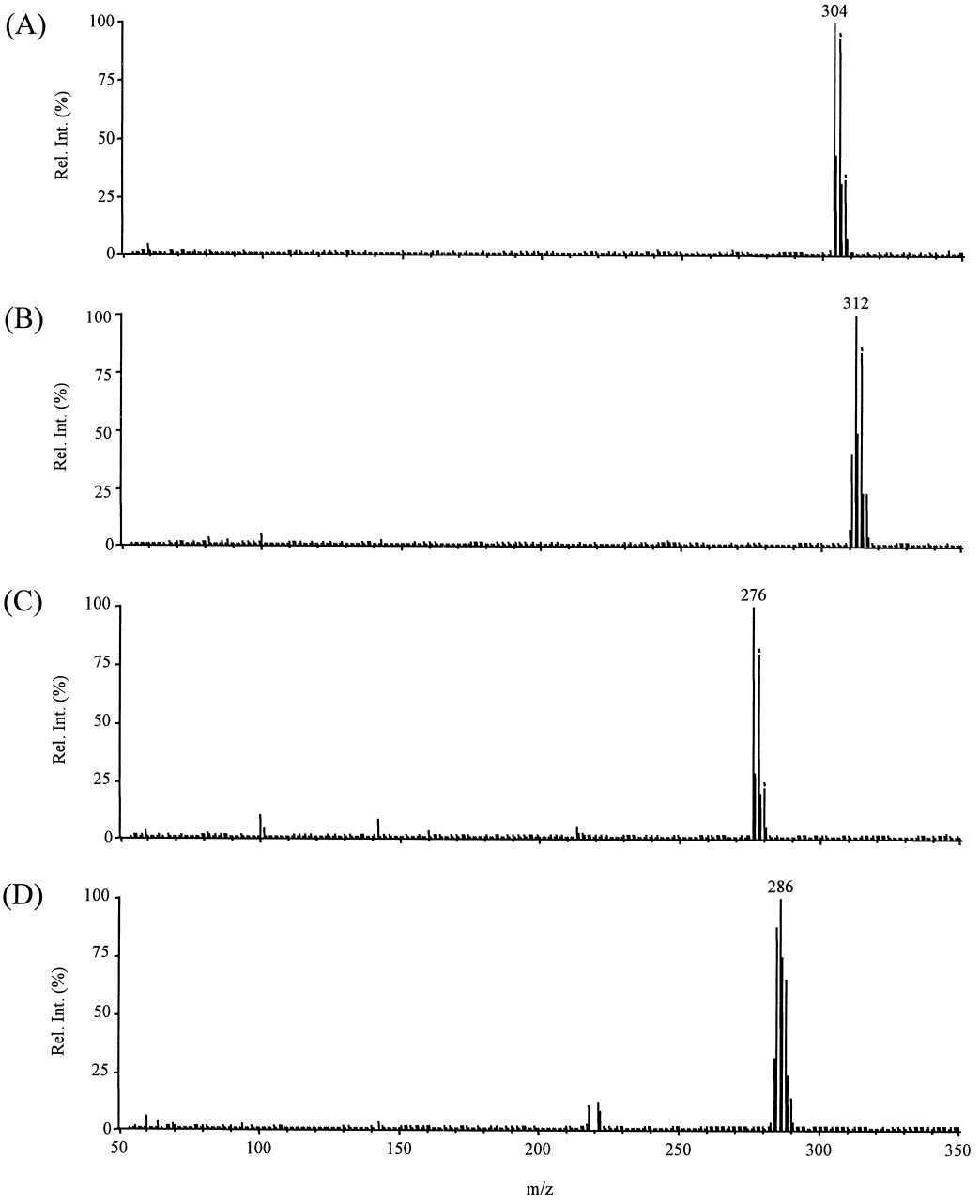

Fig. 2. Full scan LC–MS spectra of (A) chlorambucil, (B) [$^{13}C_4^2H_4$]-chlorambucil, (C) PAAM and (D) [$^{13}C_4^2H_6$]-PAAM.

200 ng/ml and 500 ng/ml, with those of equivalent unextracted solutions (n=8). The mean recovery for chlorambucil was determined to be 88.9% (SD 11.5) and for PAAM 91.5% (SD 18.6).

4.3. Method validation

The method was formally validated for chlorambucil and PAAM in human serum over the con-

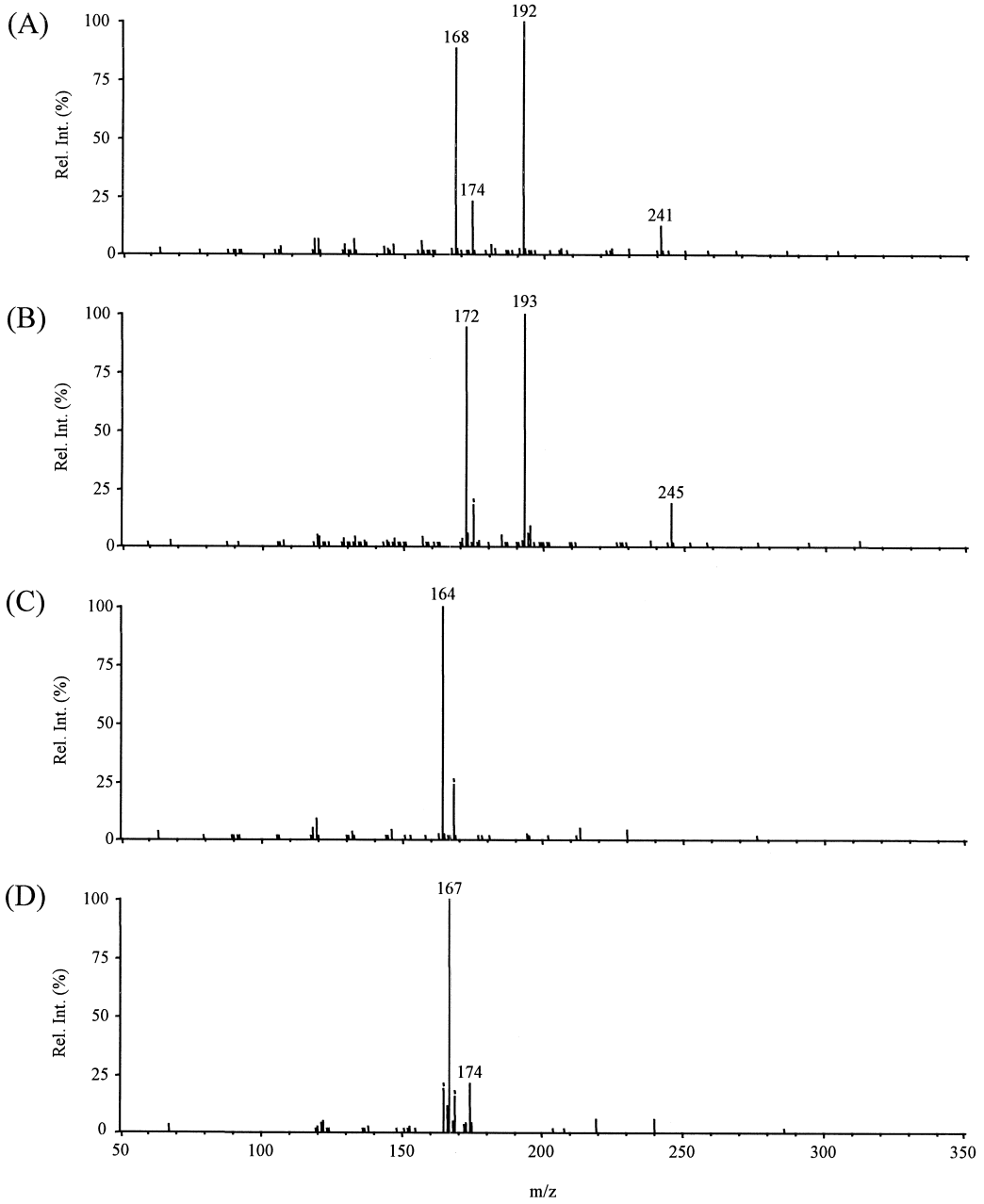

Fig. 3. Product ion scan LC–MS–MS spectra of (A) chlorambucil, (B) [$^{13}C_4^2H_4$]-chlorambucil, (C) PAAM and (D) [$^{13}C_4^2H_6$]-PAAM.

centration range 4–800 ng/ml. The precision and accuracy of the method was determined and its specificity assessed. The stability of the analytes in analytical solutions and in frozen serum (−20°C) was also determined.

The precision of the method (C.V.) was determined by assessing the agreement between replicate measurements of validation control samples prepared independently from the calibration standards. Validation control samples at four concentrations were analysed in replicates of six, on four separate occasions. The precision was also determined in human

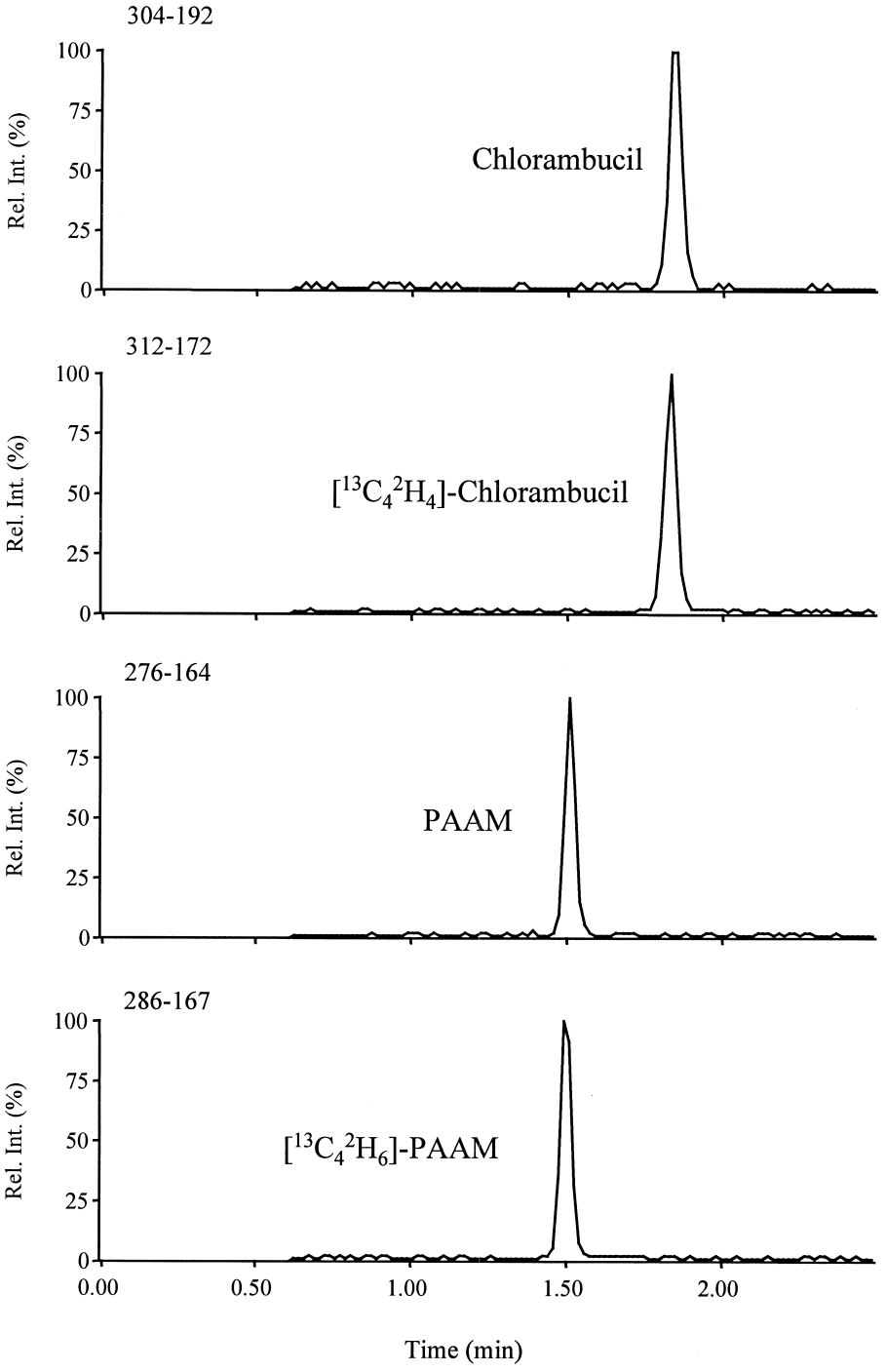

Fig. 4. SRM chromatogram of a calibration standard at 4 ng/ml.

plasma by analysing validation control samples at four concentrations in replicates of six on one occasion. Validation control samples were spiked at 4, 50, 400 and 800 ng/ml for both chlorambucil and PAAM, with the low and high concentrations determining the lower and upper limits of quantification, respectively. The data for both analytes were examined by analysis of variance (ANOVA) to give estimates of the inter- and intra-assay precision of the method. The results are presented in Table 2 and show that for both compounds, at all validation control concentrations, the precision (C.V.) does not exceed the acceptance criteria of 15% used in our laboratories [20]. The accuracy of the method (%bias) was determined in serum and plasma by assessing the agreement between the measured and actual concentrations of the validation control samples, the measured concentration being the mean of the concentrations obtained during the precision assessment. The results are presented in Table 2 and show that for both analytes, at all validation control concentrations, the bias does not exceed our acceptance criteria of ±15%. [20]

Table 2
Precision and accuracy data

Analyte	Chlorambucil			
Matrix	Serum			
Concentration (ng/ml)	4.0	50.0	400.0	800.0
n	24	24	23	24
Accuracy (%bias)	1.6	−8.5	−3.3	4.1
Intra-assay precision (C.V.)	5.2	4.3	2.5	2.3
Inter-assay precision (C.V.)	3.8	2.5	3.4	2.6
Analyte	PAAM			
Matrix	Serum			
Concentration (ng/ml)	4.0	50.0	400.0	800.0
n	24	24	23	24
Accuracy (%bias)	2.6	−0.1	−3.2	2.7
Intra-assay precision (C.V.)	5.4	5.3	2.7	3.0
Inter-assay precision (C.V.)	6.7	6.9	4.0	4.1
Analyte	Chlorambucil			
Matrix	Plasma			
Concentration (ng/ml)	4.0	50.0	400.0	800.0
n	6	6	6	6
Accuracy (%bias)	5.4	−7.4	−2.0	6.6
Intra-assay precision (C.V.)	8.8	2.6	2.9	4.7
Analyte	PAAM			
Matrix	Plasma			
Concentration (ng/ml)	4.0	50.0	400.0	800.0
n	6	6	6	6
Accuracy (%bias)	−2.4	5.4	−6.3	−5.2
Intra-assay precision (C.V.)	13.7	3.7	2.9	3.0

The specificity of the method was assessed by the visual examination of SRM chromatograms of control human matrix from several volunteers. The corresponding LC–MS–MS SRM chromatograms from an extracted control plasma sample are shown in Fig. 5. The combination of chromatographic retention time and two stages of mass selectivity, LC–MS–MS provides extremely high specificity and this is illustrated by the lack of interferences in any of the SRM traces (Figs. 4 and 5).

The stability of chlorambucil and PAAM in acidic acetonitrile analytical solution was determined by comparing peak area data obtained from a dilution of a freshly prepared solution against that of an equivalent solution that had been stored at 4°C. The stability of chlorambucil was assessed at 500 ng/ml in replicates of six and PAAM at 1000 ng/ml in replicates of eight. For the stability in biological matrix, control samples were prepared in human serum (in replicates of 6) at 750.0 ng/ml for both compounds. These were stored frozen at −20°C and after 99 days the samples were extracted and analysed together with identical but freshly prepared control samples. All stability data was compared using a two sample, one-sided *t*-test. It was concluded that chlorambucil and PAAM were stable in acidic acetonitrile solution for at least 40 days and 10 days respectively, and are stable in serum at −20°C for at least 99 days. The stability data for chlorambucil and PAAM are shown in Fig. 6.

5. Conclusion

An automated 96-well SPE and LC–MS–MS method has been successfully validated for the anticancer drug chlorambucil and its β-oxidation metab-

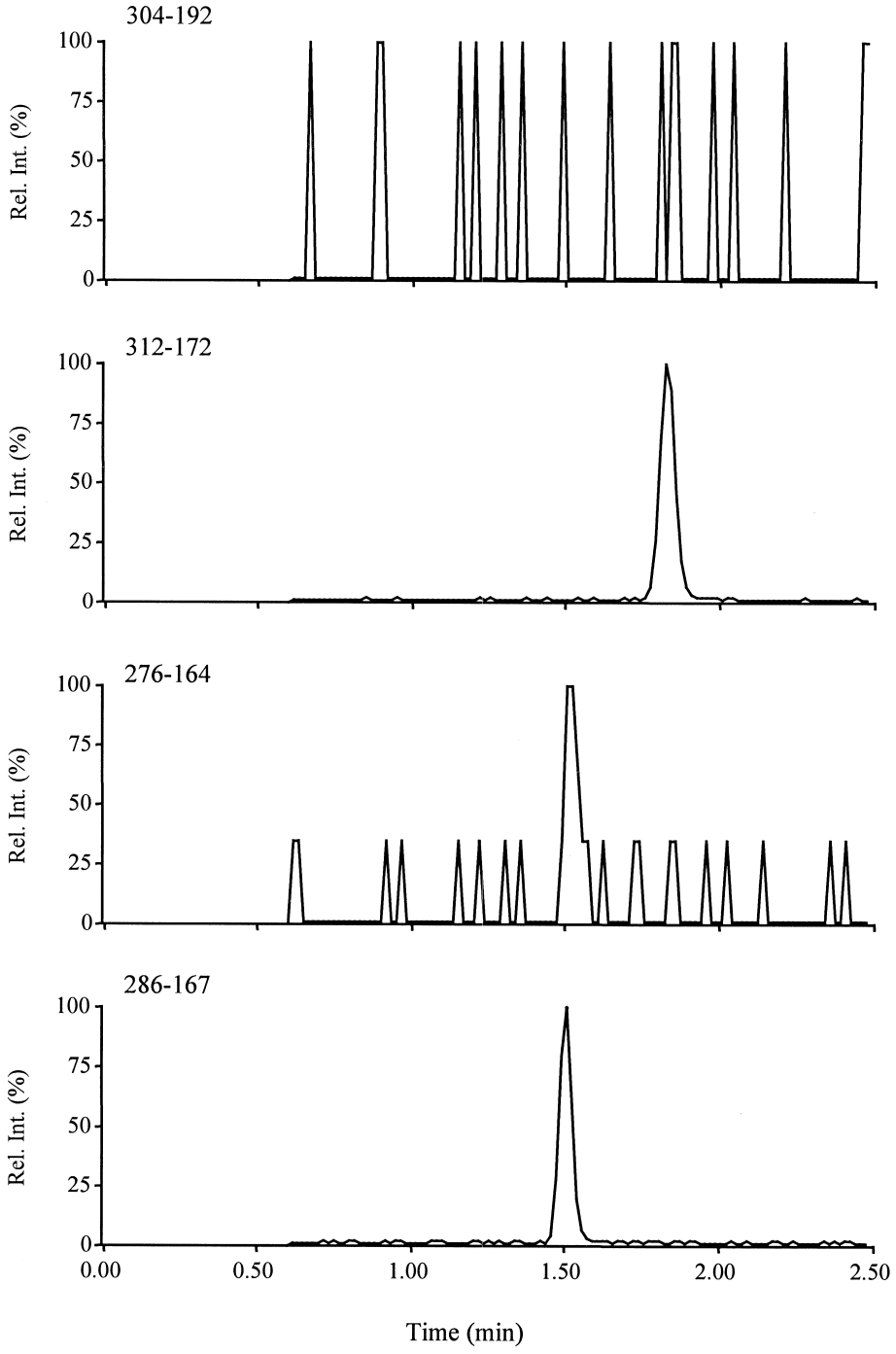

Fig. 5. SRM chromatogram of a blank matrix sample.

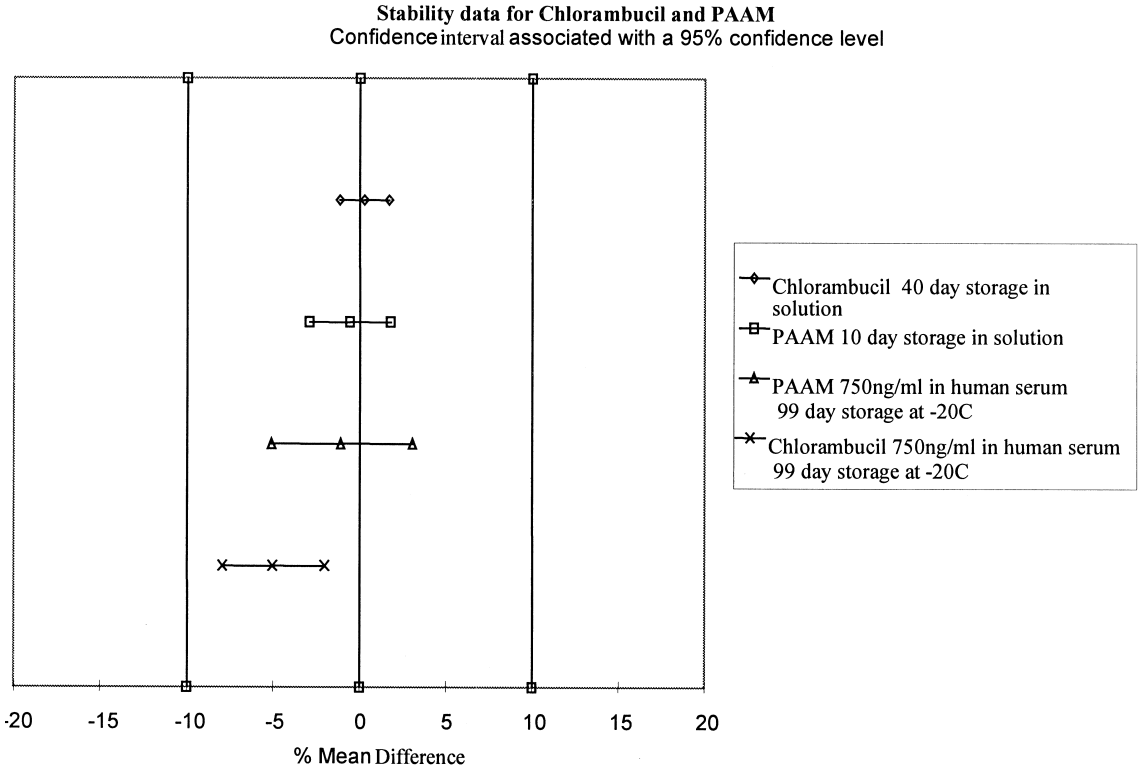

Fig. 6. Stability data for chlorambucil and PAAM.

olite PAAM in human serum and plasma. The method is specific and sensitive, providing limits of quantification of 4–800 ng/ml for parent drug and metabolite in 200 μl human serum and plasma with acceptable precision and accuracy. The method has been used to support clinical pharmacokinetic studies. Representative plasma concentration-time profiles for chlorambucil and PAAM, following a single oral dose of chlorambucil (0.2 mg/kg) from a study investigating the biocomparability of three formulations of chlorambucil (the current UK and US formulations and a new world-wide reformulation), are shown in Fig. 7. Geometric mean terminal half lives for chlorambucil for the three formulations investigated were determined to be 1.3±0.7 h, 1.1±0.3 h and 1.2±0.5 h and the corresponding values for PAAM were 1.9±0.7 h, 1.8±0.3 h and 1.8±0.4 h, respectively (n=12). These values compare reasonably well with those determined by other investigators; $t_{1/2}$ chlorambucil and PAAM 91.7±19.3 min and 145.2±29.0 min, respectively, and $t_{1/2}$ chlorambucil 86±22 min [5,6]. The geometric mean area under the curve ($AUC_{0-\infty}$) values for chlorambucil were found to be 997±524 ng·h/ml, 1001±396 ng·h/ml and 932±333 ng·h/ml for the three formulations with corresponding PAAM values approximately 1.4 times larger. Chlorambucil was measurable in the plasma after 15 min with mean t_{max} values of 1.29 h, 0.73 h and 0.83 h for the three formulations (n=12). Geometric mean peak plasma levels were 475±181 ng/ml, 575±206 ng/ml and 534±141 ng/ml for chlorambucil and 340±98 ng/ml, 381±80 ng/ml and 338±71 ng/ml for PAAM. The mean %AUC extrapolated values ranged from 1.7–2.0% (chlorambucil) and 3.5–6.3% (PAAM). The 90% confidence intervals for the ratios of geometric mean $AUC_{0-\infty}$ for the three formulations were calculated and these fell within the FDA bioequivalence range of 80–125% for each pairing for both drug and metabolite.

Using a robotic sample processor to automate the SPE, the sample preparation time of the method was

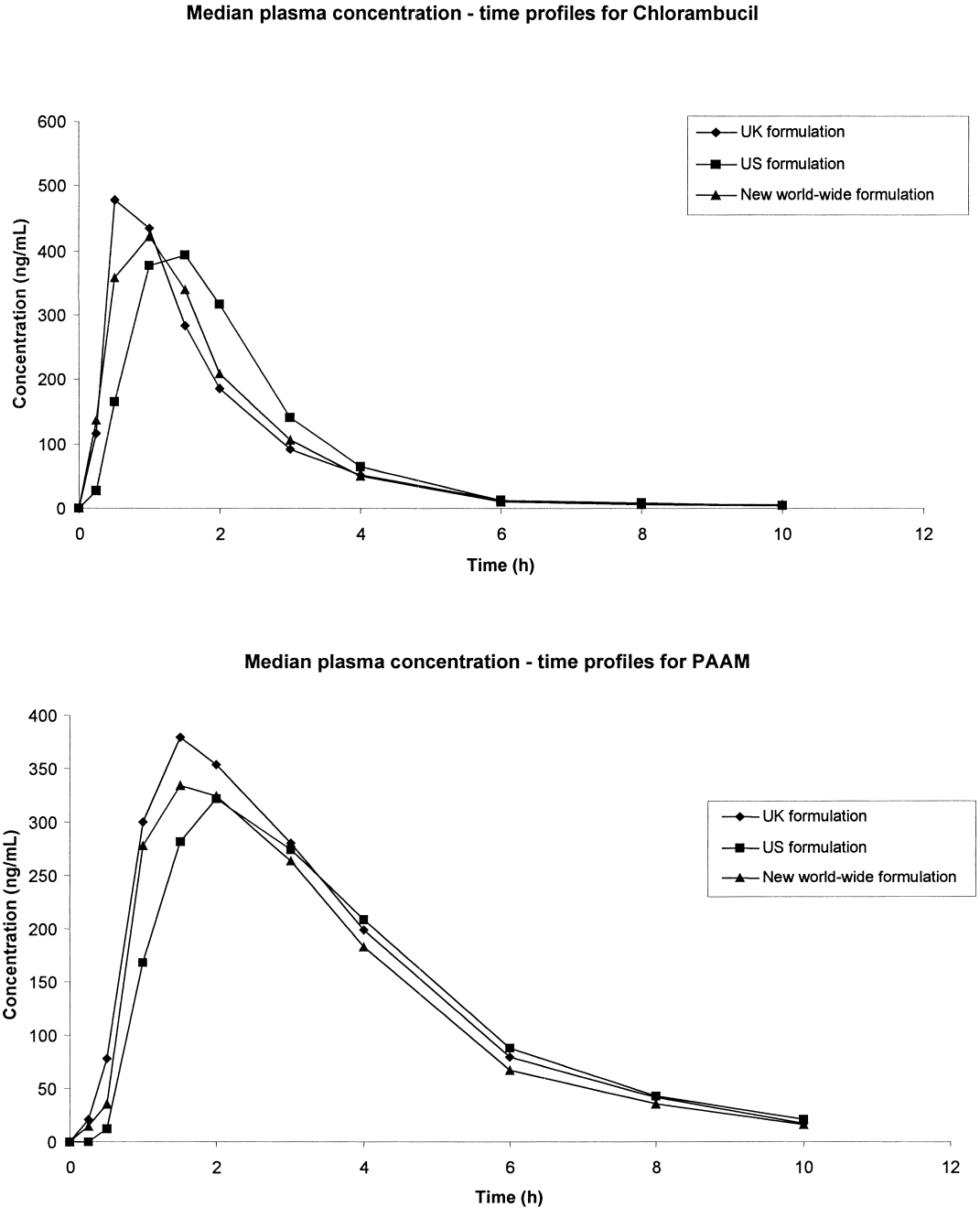

Fig. 7. Median plasma concentration-time profiles for chlorambucil and PAAM following a single oral dose of 3 chlorambucil formulations.

50 min per batch of 96 samples (74 study samples together with 14 standards, 6 quality controls and 2 blanks). With a chromatographic run time of approximately 2 min, a full batch of 96 samples can be run in 3.5 h, and this allows next day turn-around of results for study samples. In addition to speed, another advantage of the automated 96 well format over manual SPE and liquid–liquid techniques is

cost. This cost saving mainly relates directly the increase in speed giving the analyst more time to carry out other important tasks. A significant cost saving on consumables can also be incurred. The cost of a 96 well SPE block is approximately the same as individual SPE cartridges but major savings are made in the reduction of other consumables such as pipette tips, individual disposable pipettes for liquid transfers, tubes for collection, auto-sampler vials and caps. Scaling down the SPE bed volume from 100 mg (a typical SPE cartridge sorbent bed) to 15 mg also significantly reduces solvent usage. The major saving in automating the process is in freeing the analyst from laborious manual pipetting. After placing the sample racks and solvent reservoir in the pre-assigned locations on the deck of the RSP, the analyst is only required to place the collection plate under the SPE block in the manifold prior to the final elution stage.

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