Quantitation of phenylbutazone and oxyphenbutazone in equine plasma by high-performance liquid chromatography with solid-phase extraction

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Abstract

Phenylbutazone (bute) and oxyphenbutazone are non-steroidal anti-inflammatory drugs (NSAIDs) widely used in the equine world. Both substances are prohibited under the British Jockey Club Rules of Racing but are permitted up to a certain threshold level in blood plasma by some other equine governing bodies. There is therefore the requirement for accurate quantitation of these compounds in equine plasma samples.

A quantitative analytical method utilising solid-phase extraction and reversed-phase HPLC was developed and a full validation exercise performed. Additional studies of analyte stability and development of a confirmatory analysis method were also carried out.

A linear calibration over the plasma concentration range of 1–10 μg ml^{-1} for both analytes was achieved using fenclofenac as an internal marker. Inter-assay precision ($n = 6$) testing of plasma samples spiked at 2 μg ml^{-1} with both analytes produced results (R.S.D.) of 5.1% for phenylbutazone and 4.0% for oxyphenbutazone with standard error of the mean 0.0140 and 0.0138, respectively. The analytes were prone to oxidation during extraction and storage and preventative measures were incorporated into the methods. Confirmatory analysis was achieved by GC–MS with on-column derivatisation (methylation) of back extracted residues from the quantitative method.

1. Introduction

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) (PB) is one of the most widely used drugs in the equine world. It is a non-steroidal anti-inflammatory drug (NSAID) with antipyretic and analgesic activity. Its major use in the horse is in treatment of bone and joint inflammation, laminitis and soft tissue inflammation [1–8]. PB is metabolised in the liver to form oxyphenbutazone (OPB) which is pharmacologically active, and γ-hydroxyphenylbutazone which is presumed inactive [9] (Fig. 1). PB and its two metabolites have been reported to be heavily bound to plasma proteins [10] and excreted in the urine as both the unchanged drug and as the two major metabolites [11,12].

The potential misuse of NSAIDs such as PB has resulted in the banning of their use in the treatment of horses in competition by many equine governing bodies including the British Jockey Club [13,14].

PB and OPB are permitted up to a threshold plasma level by some other equestrian authorities. Therefore there is a requirement for an accurate and fully validated quantitative method for PB and OPB in equine plasma. Numerous quantitative methods for PB and its metabolites
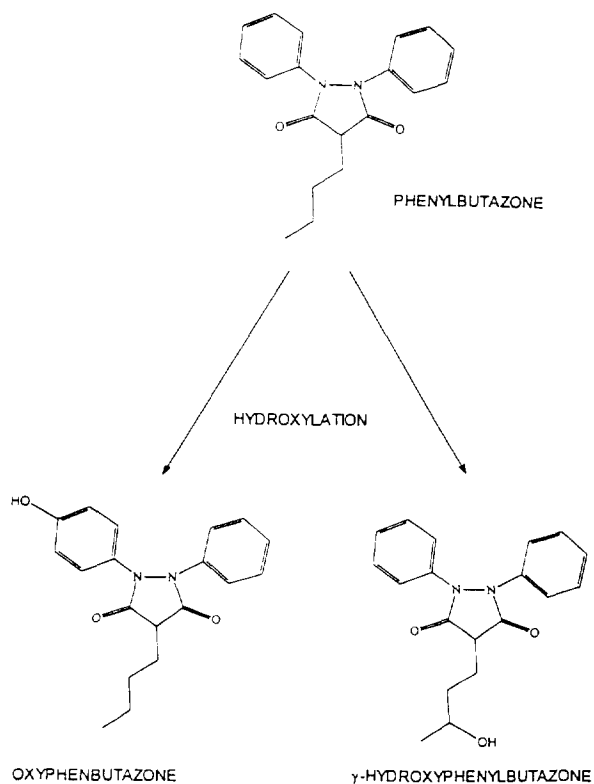
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Fig. 1. Metabolism of phenylbutazone in the horse.

2. Experimental

2.1. Materials

Isolute cartridges, C_{18} (non-endcapped, 500 mg, 6 ml reservoir) were obtained from International Sorbent Technology (Hengoed, UK). Bond elut cartridges, Certify (300 mg, 6 ml reservoir) were obtained from Anachem (Luton, UK). Diethylether, dichloromethane, ethyl acetate, hexane and methanol (HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, UK). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate and acetic acid (AnalR grade) were obtained from BDH (Poole, UK). Phenylbutazone, oxyphenbutazone hydrate, 1-heptanesulfonic acid, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and alumina were obtained from Sigma (Poole, UK). Fenclofenac {[2-(2,4-dichlorophenoxy)phenyl] acetic acid} was obtained from Reckitt and Colman (Pharmaceuticals Division, Hull, UK). Trimethylanilinium hydroxide (TMAH) (0.2 M in methanol) was obtained from Supelco (Poole, UK). Blank equine plasma was obtained from thoroughbred racehorses stabled at the Horseracing Forensic Laboratory.

have been published using both gas chromatography (GC) [15–18] and high-performance liquid chromatography (HPLC) with UV detection [19–30]. Of these only one [28] addressed the problem of decomposition of PB and OPB during extraction. There is an additional requirement to unequivocally identify the presence of PB or OPB in a positive sample (with a result greater than the threshold limit) and mass spectrometric (MS) data provides this information.

In this article we present results from a simple, fully validated quantitative analytical method for PB and OPB using HPLC with solid-phase extraction (SPE). A study of the degradation of the compounds prior to, during and after extraction has been carried out and preventative measures have been incorporated into the method. A confirmatory analysis method based upon GC–MS with a solid-phase back extraction of the HPLC sample residues is also presented.

2.2. Chromatography

The HPLC apparatus consisted of Hewlett Packard HP1090 Series I and Series II instruments with diode-array detectors and a Waters 600E multisolvent delivery system with Waters 990 photodiode-array detector and Waters WISP 712 autosampler. A monitoring wavelength of 240 nm (4 nm bandwidth) was used with reference wavelength of 550 nm (100 nm bandwidth). Peak controlled spectral data acquisition was performed over the wavelength range of 230–350 nm (2 nm step, 640 ms sampling interval). Injection volume was 50 μl.

Separations were performed on a Hypersil C_{18} column (5 μm, 100 × 4.6 mm I.D., Jones Chromatography, Hengoed, UK) under isocratic conditions at a flow-rate of 1.5 ml/min. Mobile phase was methanol (60%) and acetic acid (0.1

M) with heptane sulfonic acid (0.01%) (40%) which was filtered through 0.45 μm glass sinter and helium degassed. Chromatography was carried out at 40°C. Relative peak areas were calculated using the HPLC Chemstation integration software which was calibrated on each assay with extracted standard solutions.

The GC–MS apparatus consisted of a Varian 3400GC fitted with a Finnigan MAT A200S autosampler and Finnigan MAT Incos 50 mass spectrometer. Separations were performed on an SE54 capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm, Alltech, Carnforth, UK). Injection was made at 250°C, splitless for 70 s. Carrier gas was helium at 15 p.s.i. (103 kPA) head pressure. Temperature programme was isothermal at 100°C for 2 min then ramped at 21°C/min to 320°C then isothermal at 320°C for 7.5 min. Transfer line was maintained at 290°C, ion source at 175°C and scanning at 40–650 amu in one second.

2.3. Sample preparation

Quantitative HPLC method

Mixed standards of PB and OPB were prepared in methanol at 0.4 mg ml^{-1} calibration samples (at 1, 2, 4, 7 and 10 μg ml^{-1}) and control samples (at 2 and 6 μg ml^{-1}) in blank equine plasma. The control samples were prepared by an independent analyst using a separate mixed standard. 2 ml aliquots of calibration, control, unknown and blank plasma samples were pipetted (in duplicate) into tubes and a fenclofenac internal marker solution (0.1 mg ml^{-1} in methanol, 100 μl) were added to all except one of the blank plasma samples. Phosphate buffer (pH 7.2, 0.1 M, 1 ml) and de-ionised water (3 ml) was added to all samples before capping and mixing. Samples were drawn through C$_{18}$ cartridges which had been pre-conditioned with methanol (2 ml) and de-ionised water (2 ml) under vacuum. The cartridges were sequentially washed with phosphate buffer (pH 7.2, 0.1 M, 1 ml) and hexane (2 ml). The cartridges were dried under vacuum (approximately 20 in. Hg, ca. 68 kPA) for 2 min prior to

elution with a mixture of ethylacetate and hexane (1:1, v/v; 2 ml). The eluates were evaporated to dryness (at 40°C) under a stream of oxygen free nitrogen (OFN). The dry residues were reconstituted in methanol (100 μl) and phosphate buffer (pH 7.2, 0.1 M, 150 μl) and submitted to HPLC analysis. Low volume (i.e. < 4ml) or high concentration (i.e. ≥ 10 μg ml^{-1}) samples were diluted with blank equine plasma prior to extraction.

Confirmatory GC–MS method

Selected residues from HPLC quantitation were back-extracted for GC–MS (generally the suspicious samples, blanks and a calibration or control sample). The residues were diluted with phosphate buffer (pH 5.5, 0.1 M, 6 ml) and drawn through a Certify cartridge which had been preconditioned with methanol (2 ml) and phosphate buffer (pH 5.5, 0.1 M, 2 ml). The cartridges were sequentially washed with a methanol–phosphate buffer (pH 5.5, 0.1 M) mixture (1:9, v/v; 1 ml), acetic acid (1.0 M, 1 ml) and hexane (2 ml). The cartridges were dried under vacuum (ca. 68 kPA) for 5 min before elution with dichloromethane (3 ml). The eluates were evaporated (at 40°C) to dryness under a stream of OFN. The dry residues were reconstituted in 30 μl MSTFA and 30 μl TMAH (0.2 M in methanol) for GC–MS analysis.

3. Results

3.1. Linearity

The proportional relationship of HPLC response to analyte concentration over the working range was demonstrated. Peak areas of PB and OPB ratioed to the internal standard were plotted against concentration and subjected to linear regression analysis. The linearity was determined over the concentration range of 1 to 10 μg ml^{-1} using five data points ($n = 6$). For PB the result was $y = 4.00x + 0.0910$ ($r = 0.99$), for OPB $y = 3.29x + 0.201$ ($r = 0.999$).

3.2. Precision

The inter-assay precision, expressed as the R.S.D. was determined for PB and OPB at five concentration levels using four different operators, four different HPLC instruments and two different SPE cartridge batches. The results ($n = 6$) are presented in Table 1.

3.3. Recovery

Drug recoveries were calculated by extracting a set of calibration samples and comparing results with non-extracted standards representing 100% recovery. The ranges found were 53.5 to 63.1% for PB and 43.3 to 47.2% for OPB.

3.4. Selectivity

No significant interference peaks were observed from plasma taken from ten different horses and extracted using the quantitative procedure.

3.5. Matrix effects

Calibration samples were prepared in blank equine plasma which had been previously frozen and thawed. The results were compared to those from samples prepared in fresh (non frozen) plasma and to calibration samples prepared one month earlier and stored frozen. A negative bias in PB results obtained from fresh plasma was seen. Reductions of up to 14% were observed.

No loss of either PB or OPB was found in the sample stored frozen for one month.

The use of blank equine plasma and de-ionised water as diluents for high-concentration or low-volume samples (unknowns) was investigated. It was found that when deionised water was used as a diluent the results were negatively biased compared to when blank equine plasma was used. Depressions as high as 18% were observed.

3.6. Stability of standards

The methanolic stock standards (PB, OPB and fenclofenac) were stable for at least 8 weeks with storage at 4°C. Storage at elevated temperatures resulted in decomposition of PB and OPB (Fig. 2).

3.7. Stability during extraction

Degradation of PB and OPB was observed when diethylether was evaluated as an elution solvent in solid-phase extraction. The decomposition was reduced if the diethylether was passed through an alumina column prior to use to remove peroxides. No decomposition was observed when an ethyl acetate–hexane mixture was used as the eluent (Fig. 3).

Degradation of PB and OPB was observed if samples were exposed to acidic conditions or if samples were left dry and open to the atmosphere.

Table 1
Inter-assay precision data ($n = 6$) for quantitation of PB and OPB in equine plasma

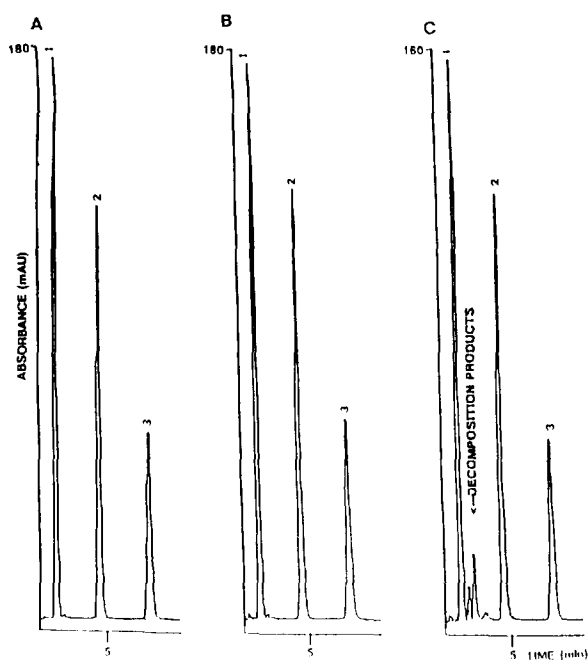| Calibration level	Concentration (μg ml^{-1})	R.S.D. (%)	
		PB	OPB
1	1	8.89	9.73
2	2	5.07	3.96
3	4	4.40	2.97
4	7	4.08	2.27
5	10	4.95	2.23

Fig. 2. Chromatograms obtained from methanolic stock standards. (A) Freshly prepared, (B) 8 weeks storage at 4°C, (C) 8 weeks storage at 37°C. 1 = OPB, 2 = PB, 3 = fenclofenac.

3.8. Stability after extraction

Plasma extracts ($n = 6$) from samples spiked at 2 μg ml^{-1} were re-analysed by HPLC after six days storage at 25°C. When results were compared to the original data no significant differences were observed.

3.9. Confirmatory analysis

Back-extracted HPLC residues from a spiked sample (4 μg ml^{-1}) were submitted to GC–MS with on-column derivatisation. The methyl derivatives of PB and fenclofenac and dimethyl derivative of OPB were identified (Fig. 4). A typical result from a post administration sample is shown in Fig. 5.

4. Discussion

The measurement of inter-assay precision during validation of the quantitative procedure is
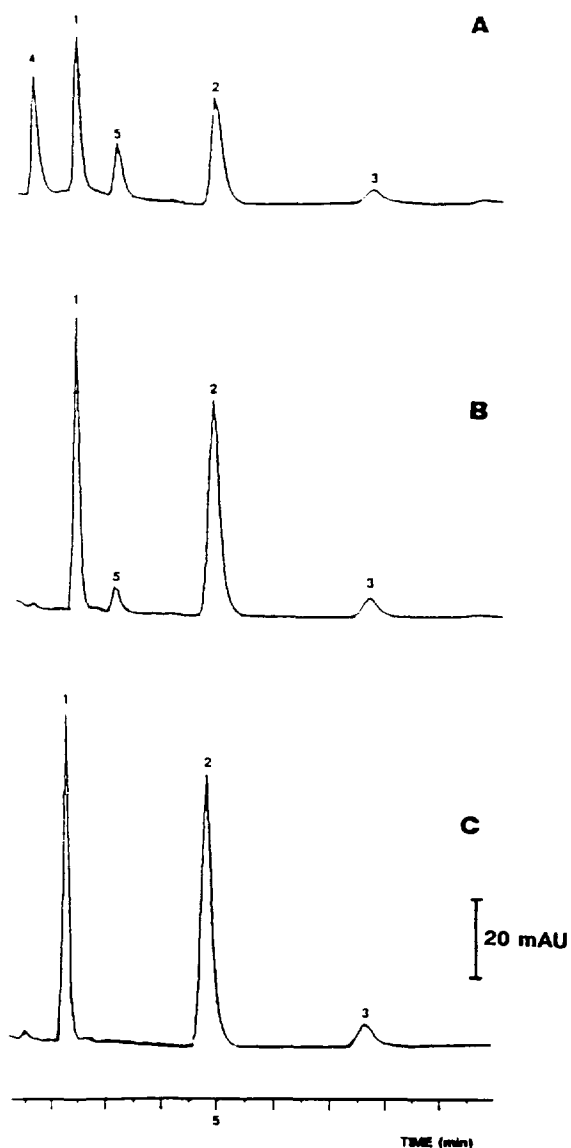

Fig. 3. Chromatograms showing degradation due to peroxide induced autoxidation of PB and OPB. Elution of C_{18} with different solvents. (A) Untreated diethyl ether, (B) alumina treated diethyl ether, (C) ethyl acetate–hexane (1:1). 1 = OPB, 2 = PB, 3 = fenclofenac, 4,5 = decomposition products.

especially important where a maximum permitted (threshold) level is applied. The validation exercise was set up to generate the maximum possible variation of results which could occur in the normal routine use of the quantitative procedure. By the incorporation of as many vari-
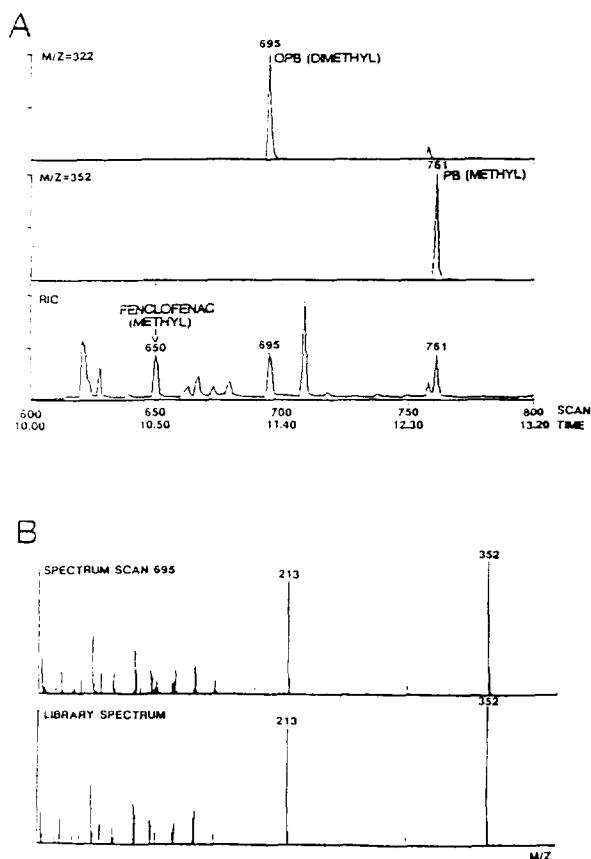
A

B

Fig. 4. Confirmatory GC–MS analysis of PB and OPB in equine plasma spiked at 4 μg ml^{-1}. (A) Reconstructed ion chromatogram and ion chromatograms, (B) OPB (dimethyl) mass spectrum with library search result.

ables as possible into the validation, i.e. different operators, HPLC instruments, SPE cartridge batches, etc., a 'worst case scenario' is created. This provides a basis upon which to decide if a result is over or under the threshold limit (within a specified level of confidence) accounting for the maximum expected level of method variation.

The problem of decomposition by oxidation of PB and OPB prior to, during and after extraction has been addressed and preventative measures incorporated into the method. Plasma was spiked with PB and OPB and frozen for 28 days prior to analysis with no indication of instability. This observation is in agreement with those made by Hyde et al. [29] who demon-

strated that sample storage and treatment prior to analysis may affect the analytical result. It is recommended that plasma is separated from the red blood cells prior to freezing, as 10–25% losses of PB and OPB may occur on thermocyling in the presence of the red blood cell fraction [29]. Whether the losses are due to drug decomposition or plasma dilution by haemolysis is unknown. We found PB and OPB to be stable in methanolic solutions for 8 weeks at 4°C whereas Carturla and Cusido [28] observed decomposition within one week under identical storage conditions. The use of diethyl ether, presumably contaminated with peroxides, during the extraction of PB and OPB was demonstrated. The authors [28] found little decomposition despite using diethyl ether in their extraction procedure. After extraction a methanol–buffer (pH 7.2) mixture was used as a sample diluent to prevent oxidation of PB and OPB which may occur if the pH is too low.

The negative bias in results from fresh (non-frozen) plasma may possibly be related to protein binding of PB. The majority of plasma samples submitted to the quantitation will have been frozen prior to analysis and thawed if routine drug screening indicated the presence of PB and/or OPB.

The GC–MS procedure was a convenient way of unequivocally identifying PB and OPB after HPLC analysis. The on-column methylation procedure was quick, simple and efficient compared to conventional derivatisation with iodomethane for example, and has been previously used in analysis of diuretics in equine urine samples [31]. The advantage of back-extraction from HPLC residues is that both quantitation and confirmation can be achieved from one aliquot of plasma rather than two separate aliquots. This is important when sample volumes are limited as is often the case when dealing with plasma.

5. Conclusion

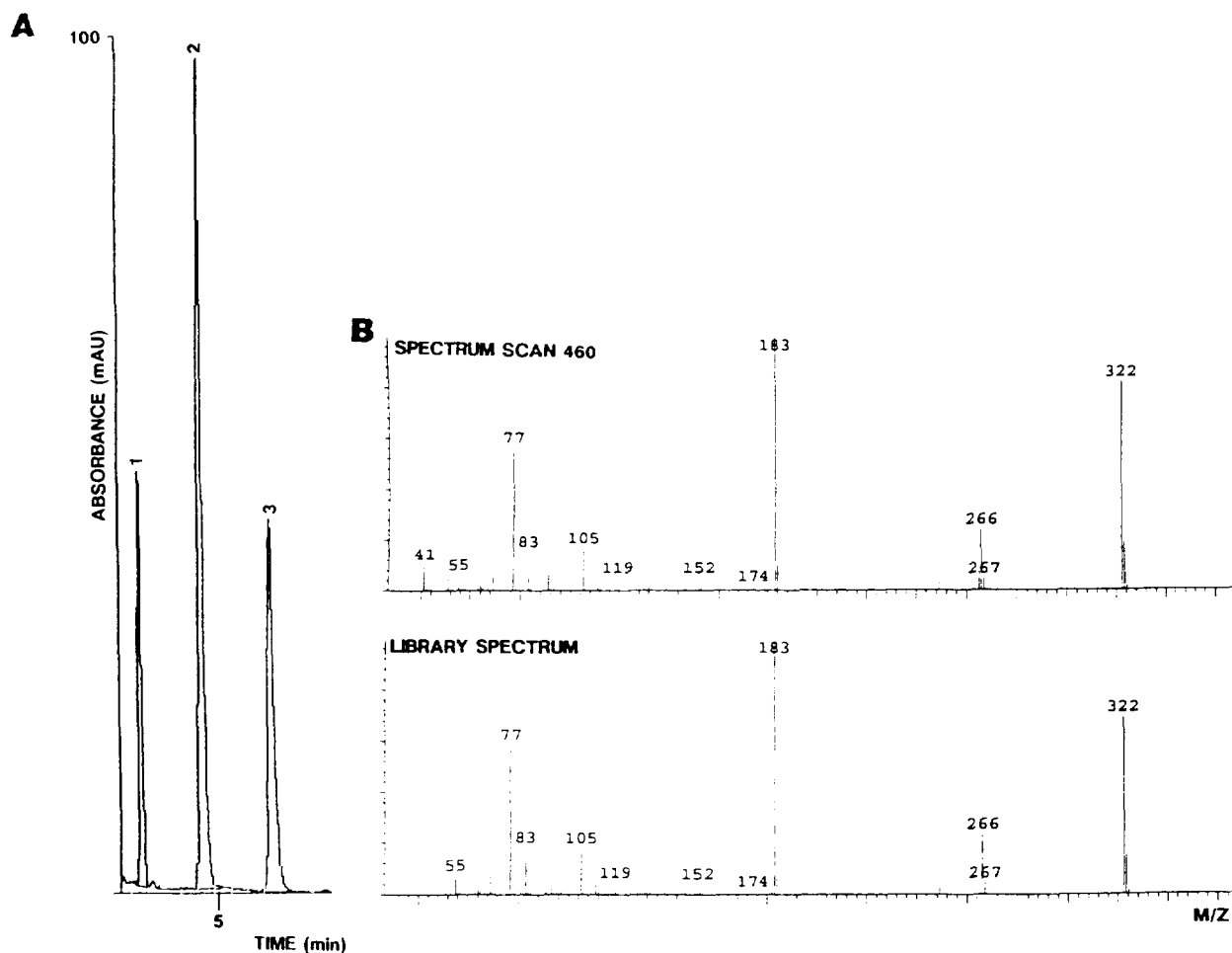An HPLC procedure with SPE has been developed and validated to measure plasma

Fig. 5. (A) HPLC chromatogram of a post administration sample. 1 = OPB (1.26 mg ml⁻¹), 2 = PB (4.61 mg ml⁻¹), 3 = fenclofenac internal standard. (B) GC–MS of positive sample and library spectrum confirming the presence of PB.

concentrations of phenylbutazone and oxyphen-butazone in equine samples. The assay is linear over the range of concentrations tested and has a lower limit of quantitation below 1 μg ml⁻¹ using 2 ml plasma. The quantitative procedure avoids decomposition of the compounds before, during and after extraction and HPLC.

A GC–MS confirmatory procedure utilising on-column methylation was developed and was successfully applied to the HPLC residues after solid-phase back extraction.

The sample preparation procedures are simple and have potential for automation.

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