ISOLATION OF DRUG RESIDUES FROM TISSUES BY SOLID PHASE DISPERSION

STEVEN A. BARKER*, AUSTIN R. LONG and CHARLES R. SHORT

Department of Physiology, Pharmacology and Toxicology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803 (U.S.A.)

(First received January 17th, 1989; revised manuscript received April 17th, 1989)

SUMMARY

A new method based on solid phase dispersion of tissue for the subsequent isolation of drugs is reported. By blending tissues with a polymeric phase bound to a solid support one obtains a semi-dry material which can be used as a column packing material from which one can isolate drugs in a stepwise fashion based on the solubility characteristics of the drugs in this matrix. The applicability of this approach for multidrug residue extraction from a single sample is demonstrated for compounds representing the organophosphate, benzimidazole anthelmintic and β-lactam antibiotic drug classes.

INTRODUCTION

The isolation of drug and metabolite residues from tissues is often a complex and laborious task. This is due to the nature of the matrix, containing significant quantities of connective tissues, proteins, lipids, etc., of a compartmentalized and difficult to disrupt nature. Classical methology has, in general, approached the isolation of drugs and metabolites from this matrix in the following manner: (1) mincing and/or mechanical homogenization of the tissue in an aqueous solvent; (2) addition of acids, bases or salts to precipitate protein and remove cellular debris; (3) centrifugation; (4) transfer of the supernatant and adjustment of pH; (5) counter-current extraction of the sample, often leading to intractable emulsions; (6) back-extraction to assist in purification of the sample. Homogenization in and repeated extraction of tissues by organic solvents has also proven to be a useful approach but generates large volumes of solvents which must be evaporated and usually requires back-extraction[1,2].

A portion of this labor may be eliminated by the use of solid phase extraction (SPE) columns wherein a supernatant, obtained as described through step 4 above for example, is added to a SPE column appropriate for the analysis and the compounds of interest are isolated from other sample components based on interaction with the column polymer phase. This process eliminates emulsion formation and much of the sample manipulation required by classical methods (for example see refs. 3–7). How-

ever, the process of sample homogenization, centrifugation, precipitation, etc., remains labor intensive and contributes, through sample manipulation and the entrainment or electrostatic binding of some drugs to tissue debris, to lower than ideal recoveries.

We present here a new approach to the isolation of drugs and their metabolites from tissues that appears to eliminate many of these difficulties. This approach employs the use of a lipophilic solid phase packing material (C_{18}) to disperse tissues onto a solid support and, thus, to produce a semi-dry, easy to handle column packing material from which individual drugs, a class of drugs or several classes of drugs may be isolated from a single sample. This approach and the underlying concepts are examined using spiked tissues.

EXPERIMENTAL

Bulk C_{18} (octadecylsilane-coated silica bead, 40 μm, end capped, 18% load) SPE column packing material was obtained from Analytichem, Harbor City, CA, U.S.A. The material was washed prior to use by placing 24 g of the packing in an emptied 50-ml Chem-Elut column (Analytichem), attaching the column to a vacuum box and successively adding 50 ml each of HPLC grade hexane, benzene, ethyl acetate and methanol to remove contaminants inherent in manufacture. Tissues (bovine muscle) were obtained from commercial food markets and were kept frozen at $-5°C$ until utilized. Thawed tissues (0.5 g) were injected (10-μl syringe) with drugs dissolved in dimethyl sulfoxide or dimethylformamide at various concentrations for analysis. Blank samples were injected with the corresponding solvents used to dissolve the drug standards.

The following classes of compounds were examined: (1) organophosphates (phenthion, crufomate, coumaphos and famphur); (2) benzimidazole anthelmintics [fenbendazole (FBZ), oxfendazole (FBZSO), sulfonyl FBZ (FBZSO$_2$), p-hydroxy-FBZ (FBZOH), mebendazole (MEB), thiabendazole (THI) and albendazole (ALB)]; (3) β-lactam antibiotics (penicillin, ampicillin and cephapirin). The concentrations examined are shown in Table I.

Sample preparation

Samples were prepared in the following manner: Tissue blanks or spiked tissues (0.5 g) were added to 2.0 g of prewashed C_{18} packing material in a glass mortar. The sample was gently blended with a glass pestle for 30 s to produce a semi-dry, homogenous appearing material. This was added to a syringe barrel-column (10 ml) containing a frit (0.45 μm) and 0.50 g of clean C_{18} packing at the bottom. Clean packing (0.25 g) was added to the top of the column and the column was lightly tamped to remove air pockets. A retainer was placed on top of the material and a syringe plunger was used to compress the sample to a volume of 4 ml. A 100-μl disposable pipette tip was attached to the end of the column and the column was placed in a rack, ready for elution (see Fig. 1). The following elution profile, collecting each fraction separately, was performed; hexane, benzene, ethyl acetate and methanol (8 ml of each, respectively). The four fractions were evaporated to dryness under dry nitrogen and an appropriate solvent was added to solubilize the residue. (1) Hexane and benzene fractions, 500 μl n-hexane. (2) Ethyl acetate fraction, 50 μl acetonitrile and 450 μl of

TABLE I

LIST OF COMPOUNDS EXAMINED

Range of concentrations (μg/g) analyzed, eluting solvent wherein drug was obtained, correlation coefficients (r, $\pm$ standard deviation, S.D., 6 points) for standard curves, recoveries (calculated for all concentrations, $\pm$ S.D., inter- and intra-assay variabilities (mean of n determinations). Data were obtained as described in Experimental.

Compound	Fraction collected	Concentration (μg/g) range examined	$r \pm$ S.D. ($n = 4$)	Recovery $\pm$ S.D. ($n = 20$)	Inter-assay variability ($n = 20$)	Intra-assay variability ($n = 5$)
Fenthion	Hexane	0.1–2	0.997 $\pm$ 0.003	85.62 $\pm$ 7.50	8.40	3.01
Coumaphos	Hexane	0.1–2	0.998 $\pm$ 0.007	76.57 $\pm$ 7.87	20.62	7.08
Famphur	Benzene	0.4	–	82.10 $\pm$ 8.78	10.70	5.50
Cruformate	Benzene	0.1–2	0.992 $\pm$ 0.006	93.64 $\pm$ 6.38	6.82	6.05
Thiabendazole	Ethyl acetate	0.2–4.0	0.9975 $\pm$ 0.0019	63.82 $\pm$ 9.57	6.91	2.74
FBZSO	Ethyl acetate	0.2–4.0	0.9912 $\pm$ 0.0019	82.86 $\pm$ 9.48	7.08	3.79
FBZOH	Ethyl acetate	0.2–4.0	0.9900 $\pm$ 0.0081	68.35 $\pm$ 10.5	13.31	8.18
FBZSO$_2$	Ethyl acetate	0.2–4.0	0.9962 $\pm$ 0.0009	85.67 $\pm$ 15.04	5.01	3.01
Mebendazole	Ethyl acetate	1.0	–	63.01 $\pm$ 4.24	8.10	4.37
Albendazole	Ethyl acetate	0.2–4.0	0.9975 $\pm$ 0.0017	73.92 $\pm$ 7.99	3.07	3.95
FBZ	Ethyl acetate	0.2–4.0	0.9842 $\pm$ 0.0099	73.97 $\pm$ 11.82	7.29	5.65
Cephapirin	Methanol	0.2–5	0.992 $\pm$ 0.007	72.37 $\pm$ 26.48	10.23	6.90
Penicillin	Methanol	0.2–5	0.994 $\pm$ 0.005	86.29 $\pm$ 6.12	17.88	4.99
Ampicillin	Methanol	2.0	–	59.75 $\pm$ 9.75	27.82	8.61

0.05 N phosphoric acid. The sample was vortexed and filtered through a 0.45-μm disposable filter (Bio-Rad). (3) Methanol fraction, 500 μl of 0.05 N phosphoric acid. The resulting suspension was centrifuged (5 min at 17 000 g) and the supernatant was filtered through a 0.45-μm disposable filter. The compounds detected in each fraction are shown in Table I.

Sample analysis

Organophosphates. Analyses were conducted by gas chromatography (GC) using a Varian Vista 6000. Column, DB-5 (J & W Scientific), 25 m × 0.25 mm I.D., 0.25-mm coating. Temperature program, 150°C for 1 min, increasing at 10°C/min to 300°C and holding for 2 min. Splitless injection was used with the purge function being activated at 0.75 min post injection. Injection port temperature, 250°C. Detection was accomplished using a nitrogen–phosphorus detector at 300°C, 6.0 mV at 10^{-12} sensitivity setting.

Benzimidazoles. Analyses were conducted by high-performance liquid chromatography (HPLC) with photo diode-array detection (Hewlett-Packard 1090). Col-
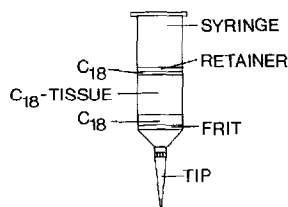

Fig. 1. Representation of a column constructed for conducting analyses as described.

umn, octadecylsilane, 12.5 cm × 0.5 cm I.D., 10 μm particle size (Varian Assoc., MCH-10). Solvent system, isocratic (0.75 ml/min), 0.05 N phosphoric acid–acetonitrile (67:33, v/v). Column temperature, 45°C. Detection and quantitation were conducted at 290 nm (20 nm band width, reference spectrum range of 200–350 nm). Full UV spectra were used to determine, in part, the identity of each benzimidazole and the purity of each peak.

β-*Lactams.* Analyses were conducted as described above using a detection wavelength of 230 nm and a solvent system (isocratic, 1.0 ml/min) of 0.05 N phosphoric acid–acetonitrile (80:20) and a column temperature of 35°C.

Recoveries for all compounds were determined from comparison of the data obtained from extracted samples to the data obtained by direct analysis of each compound at the respective concentrations without extraction.
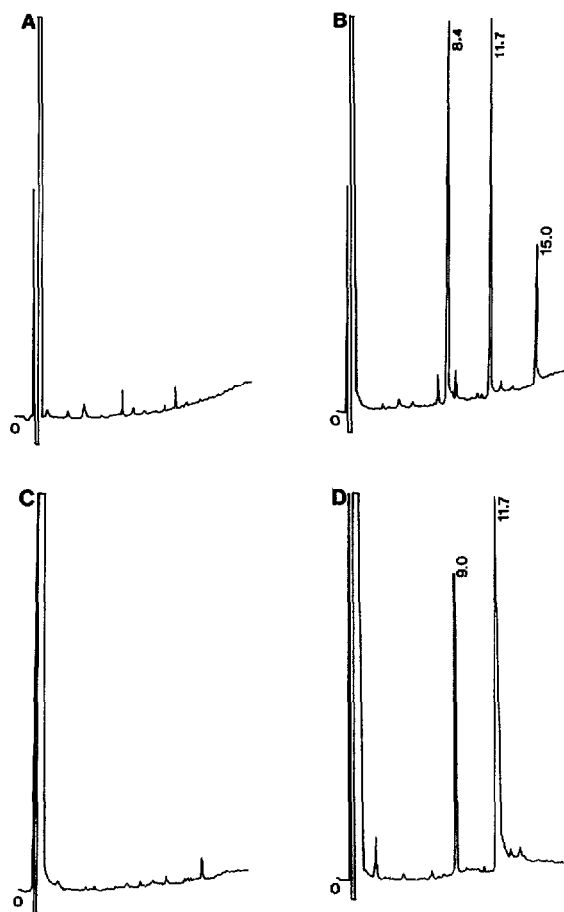

Fig. 2. Representative gas chromatograms from the analysis of the solvent eluate fractions obtained as described in Experimental. (A) Tissue blank, hexane fraction; (B) hexane fraction from tissue spiked with the drugs listed in Table I, showing the presence of phenthion (8.4 min, 0.4 μg/g), famphur (added as an external standard at 0.4 μg/g of sample, 11.7 min) and coumaphos (15.0 min, 0.4 μg/g); (C) tissue blank, benzene fraction; (D) benzene fraction from spiked tissues showing the presence of crufomate (0.1 μg/ml, 9.0 min) and famphur (internal standard, 0.4 μg/ml, 11.7 min).

RESULTS

Table I shows the levels of each compound examined, the fraction in which it was obtained, the recoveries for each compound, correlation coefficients of standard curves and the inter- and intra-assay variability obtained for analyses so conducted. Fig. 2A–D illustrate the GC–nitrogen–phosphorus detection analyses of blank tissue extracts obtained from the hexane and benzene eluates (A and C, respectively) and that obtained for spiked tissues (B and D, respectively). The organophosphates phenthion and coumaphos were detected in the hexane fraction, whereas the compounds crufomate and famphur were observed to elute in the benzene fraction. Famphur was used as an internal standard in these studies, being spiked in all samples prior to extraction at a level of 0.4 $\mu g/g$ and was added as an external standard for the analysis of the hexane fraction.

The presence of a small quantity of lipid was noted in the hexane and benzene fractions but its presence did not interfere with the analyses of the organophosphates in any manner. Analyses of the hexane and benzene fractions by HPLC gave no indication of the presence of even trace levels of the benzimidazole anthelmintics. No further sample cleanup was required for the analysis of the organophosphates in these fractions.

Analysis of the ethyl acetate fraction by HPLC (Fig. 3A–B) showed that all of the benzimidazoles examined here are eluted in this fraction. The compound mebendazole (MEB) served as an internal standard for these analyses. Under the conditions used no interfering substances were noted, eliminating the need for back-extraction or further cleanup of the sample prior to analysis. Analysis of the ethyl acetate fraction by the HPLC method described for β-lactams gave no indication that the β-lactams
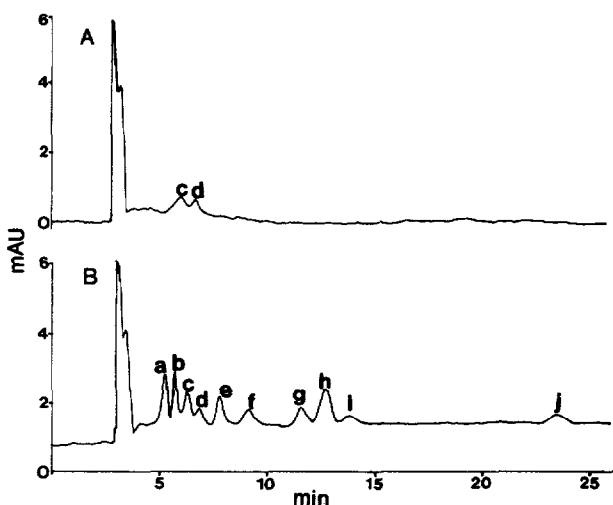

Fig. 3. Representative chromatograms obtained from the HPLC–diode-array analysis of the ethyl acetate eluate fraction of (A) a tissue blank and (B) from that obtained from a spiked (0.2 $\mu g/g$) tissue. Peaks: a = THI (retention time 5.2 min), b = unknown sample component (5.6 min), c and d = background contaminants (6.2 and 6.7 min), e = FBZSO (7.7 min), f = FBZOH (9.0 min), g = $FBZSO_2$ (11.5 min), h = MEB, internal standard (1.0 $\mu g/g$) (12.5 min), i = ALB (13.6 min), j = FBZ (23.4 min).
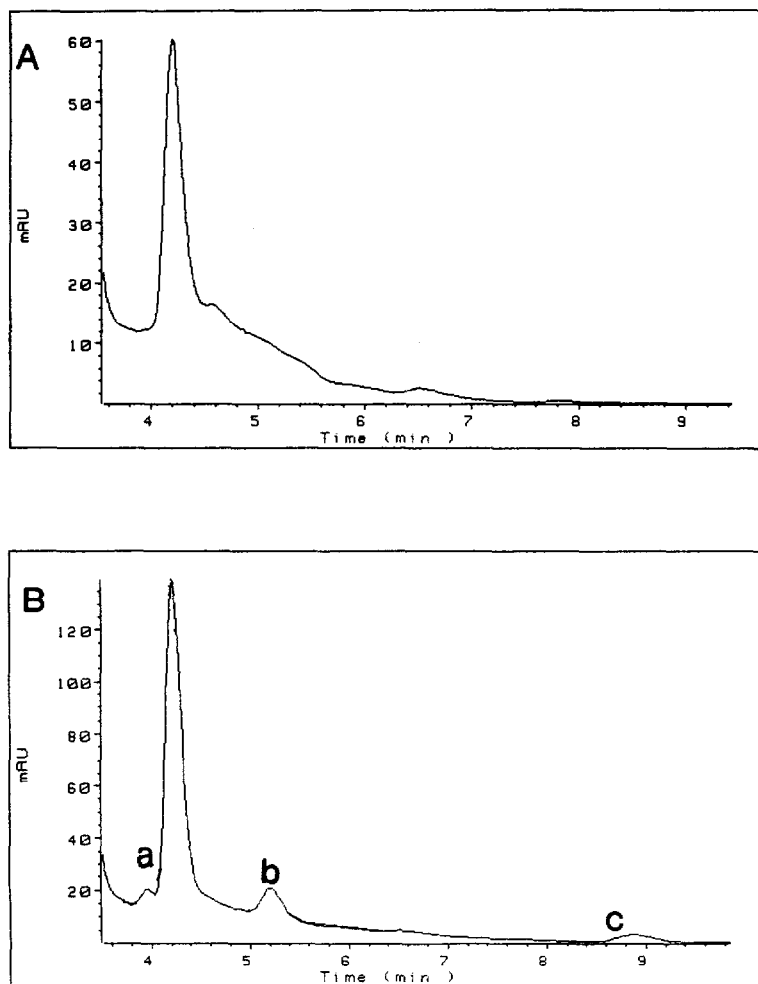
Fig. 4. Representative chromatograms obtained from the HPLC–diode-array analysis of the methanol eluate fraction from (A) a tissue blank and (B) from that obtained from a spiked (1.0 μg/g) tissue sample. Peaks: a = cephapirin; b = ampicillin (internal standard, 2.0 μg/g); c = penicillin.

are eluted in this fraction. Elution of the columns with methanol yielded, upon evaporation, a white residue which, upon testing with ninhydrin, was determined to consist mainly of proteins. Addition of 0.05 N phosphoric acid, vortexing and centrifugation (17 000 g) of the resulting suspension produced a relatively clean supernatant, low in proteinaceous material. Analysis of the resultant samples by HPLC (Fig. 4A–B) indicated that, for the analysis of penicillin and cephapirin, using ampicillin as an internal standard, no further sample cleanup was necessary. Analysis of the methanol extract by the method for benzimidazoles gave no evidence for the presence of any of the benzimidazoles in this fraction.

DISCUSSION

The application of SPE technology to the isolation of a specific drug or class of drugs from various matrices has grown tremendously in the last decade. Much of this growth has been due to the relative ease of sample handling with samples being poured directly to the column with little or no prior preparation, and the wide range of polymer phases bound to solid supports currently available for a variety of applications. Further, elution profiles for the compound(s) of interest from the matrix employed has a high degree of flexibility and can be readily varied for a given analytical problem. The use of SPE also avoids the formation of emulsions, a common occurrence with counter-current extraction, and, in most cases, reduces the volume of solvents required for efficient isolation of the compound(s) of interest. However, the use of SPE for the isolation of drugs, environmental contaminants and natural compounds from tissues has heretofore required sample homogenization and removal of tissue debris prior to column application. Addition of homogenates directly to the top of a column invariably leads to the cessation of flow from the plugging of the frit or upper layers of the column packing. We have previously reported a partial solution to this problem through the blending of homogenized tissues with diatomaceous earth (DE), for the isolation of the benzimidazole anthelmintics and their metabolites from liver tissue[8] as well as other matrices[9]. By mixing dry DE with homogenized tissues one obtains a semi-dry column packing material from which the benzimidazoles can be eluted with ethyl acetate. Through mixing the homogenate with DE one eliminates the need for precipitation of cellular components and centrifugation of the sample to pellet the debris. Further, the surface area of the sample exposed to the solvent is increased and the entire sample, proteins, connective tissue, etc., is exposed to extractive elution. However, we have observed that the use of DE is limited in application in terms of providing a more generic matrix from which several classes of compounds can be isolated from a single sample. While neutral and moderately polar compounds can be isolated from the DE matrix, compounds of greater polarity (β-lactams for example) are highly retained. This property has advantages of its own but does not truly provide a multi-residue extraction capability.

Classical methodologies have on occasion employed the use of surfactants or detergents to disrupt or, otherwise, dissolve tissue cell membranes so as to liberate internal components of cells and to remove protein and other cellular components from the lipid membrane matrix. The use of such detergents often leads to their interfering in the isolation procedure or subsequent analysis and additional steps must often be taken to assure their removal, which can also be a laborious task. The approach presented here can be seen as an extension of this concept; the use of a lipid solubilizing material to disrupt cell membranes and to, essentially, disperse tissues, but with the dispersing agent being bound to a solid support[10]. We envision this process as disrupting the cell membrane through solubilization of the component phospholipids and cholesterol into the C_{18} polymer matrix, with more polar substituents directed outward, perhaps forming a hydrophilic outer surface on the bead. If this is the case then the process could be viewed as essentially turning the cells inside-out and forming an inverted membrane with the polymer bound to the solid support. This process would create a pseudo-ion exchange/reversed phase for the separation of added components. Thus, the C_{18} polymer would be modified by cell

membrane phospholipids, interstitial fluid components, intracellular components, cholesterol, etc., and would possess elution properties that would be dependent on the tissue used, the ratio of C_{18} to tissue employed and the elution profile performed. We have observed this to be the case in our preliminary examination of other tissues, wherein the elution profile is altered by the type of tissue used and may be further modified by changing the ratio of C_{18} to tissue.

Preliminary examinations of the blended materials by scanning electron microscopy indicate that complete disruption of the cells does occur and that this process of disruption may be further modified by the use of a solvent to blend the tissues, as determined from preliminary studies.

The blending of tissue with C_{18}-coated silica beads by the use of a mortar and pestle proceeds rapidly and smoothly, producing a semi-dry, homogenous appearing material. This has been observed to be the case with either fat, liver or muscle tissues. The mechanical forces applied during homogenization may be sufficient to lead to fracturing of some of the beads. However, to which degree this may occur, it does not appear to effect the flow of solvent through the column or lead to active sites wherein compounds may be lost. The effect of active site formation may not become evident except at lower concentrations (< 100 ng/g) and may limit the extension of this approach.

The range of compounds examined here lends credence to the proposition that this approach may provide a generic technique for multiresidue analysis of drugs and their metabolites in tissues. Further, this approach eliminates the need to conduct tissue homogenization, precipitation, centrifugation, pH adjustments and sample transfers. While examined here as a multiresidue approach, the results imply that such a methodology may be applied to a specific class of compounds or a single compound, whether of exogenous or endogenous origin. We have further observed that tissue "dissolution" onto a solid support can also be conducted with C_3, C_8, C_3 sulfonic, as well as other polymer phases, with the major criterion being the presence of a lipid solubilizing polymer phase. Each of the various phases available may be useful in more specific applications. The concept of blending a sample with a solid support, producing a column packing material unique to each sample matrix, is also feasible, such as the use of microcrystalline cellulose to blend and extract cellulosic materials.

In the case of the drugs examined here, little or no further sample cleanup was necessary prior to analysis. This may, of course, not be the case for other drugs or for other tissues and depends, in part, on the compound to be isolated and the instrumentation used for detection. Nevertheless, a major portion of the isolation of a compound from tissue may be performed by this technique and be followed by appropriate back-extraction or other cleanup steps to sufficiently purify the sample prior to analysis.

The results presented here are based on spiked tissues, much as would be required or obtained for the preparation of standard curves or for conducting recovery studies, for the quantitative analysis of tissue residues incurred from the administration of these drugs. The purpose of the present research was to demonstrate the application of solid phase dispersion for the isolation of the fourteen drugs examined here from a single sample. While the use of tissues from animals actually administered these drugs would be ideal it was outside the scope and practicality of the present

research. Such studies are currently being conducted on these and other classes of compounds seperately, examining incurred residues in muscle as well as other tissues obtained from animals used in drug depletion studies, with the assistance of the United States Food and Drug Administration.

This approach may be applied to the isolation of drugs and their metabolites from edible tissues, as occurs in food safety monitoring programs, for toxicological examination of *post-mortem* tissues for drugs, or for the isolation of naturally occurring compounds from various tissue sources. The adaptability of the method, in terms of the stated variables, should make this approach useful for these and other applications.

REFERENCES

1 F. Tishler, J. L. Sutter, S. N. Bathish and H. F. Hagman, *J. Agr. Food Chem.*, 16 (1968) 50–53.
2 W. Horowitz, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 104–130.
3 G. Musch and D. L. Massart, *J. Chromatogr.*, 432 (1988) 209–222.
4 F. T. Delbeke, M. Debackere, N. Desmet and F. Maertens, *J. Chromatogr.*, 426 (1988) 194–201.
5 S. H. Wong, *Clin. Chem.*, 34 (1988) 848–855.
6 C. T. Wehr, *J. Chromatogr.*, 418 (1987) 27–60.
7 R. W. Giese, *Clin. Chem.*, 29 (1983) 1331–1343.
8 S. A. Barker, T. McDowell, B. Charkhian, L. Hsieh and C. R. Short, *J. Assoc. Off. Anal. Chem.*, submitted for publication.
9 S. A. Barker, L. Hsieh and C. R. Short, *Anal. Biochem.*, 155 (1986) 112–118.
10 S. A. Barker, A. R. Long and C. R. Short, in W. Huber (Editor), *Proceedings of the 6th Biennial Symposium of the American Academy of Veterinary Pharmacology and Therapeutics, Blacksburg, VA, June 1988*, American Academy of Veterinary Pharmacology and Therapeutics, Blacksburg, VA.