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Simple and rapid method for the determination of the diastereomers of difenacoum in blood and liver using high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A rapid and sensitive high-performance liquid chromatographic method for the analysis of *cis* and *trans* diastereomers of the anticoagulant rodenticide difenacoum has been described. The methodology demonstrates potential for the analysis of diastereomers of related 4-hydroxycoumarin anticoagulants. Separations were achieved by reversed-phase chromatography on a Zorbax ODS column with gradient elution using acetonitrile–water, modified with 0.1% acetic acid, as the mobile phase. Detection of the analytes was effected by fluorescence at excitation and emission wavelengths of 310 and 390 nm, respectively. Sample preparation from both plasma and liver has been simplified to reduce preparation time and manipulation. The minimum detectable concentration of each diastereomer was 5 ng/ml. Recoveries of 100% were obtained from plasma and 93% from liver tissue. This method has been used for the investigation of the pharmacokinetics of difenacoum diastereomers in rats, and for investigation of unexplained hypoprothrombinaemic events encountered clinically.

INTRODUCTION

Techniques for the determination of anticoagulant rodenticides from animal tissues [1–3] have been designed to identify the presence of one or more of a range of unknown anticoagulants simultaneously. These methods employ complex, lengthy, extraction and clean-up procedures prior to analysis, dictated by the nature of the tissues under investigation. Warfarin [3-(3-oxo-1-phenylbutyl)-4-hydroxycoumarin] and structurally related second-generation anticoagulants are commercially available for use as rodenticides as mixtures of enantiomers and/or diastereomers. Previous methods have been described for the separation of diastereomers [1,3,4] of second-generation anticoagulants, but unfortunately the diastereomers were not identified in any of those papers. The enantiomers of warfarin have different toxicities in rat [5] and man [6]. It is possible that the differences in toxicity between the diastereomers of structurally similar 4-hydroxycoumarin anticoagulants may be related to differences in their absorption, distribution, elimination or metabolism. In order to investigate the pharmacokinetics of the diastereomers (Fig. 1) of the second-generation anticoagulant difenacoum (3-[3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin), and potentially other structurally related anticoagulants brodifacoum (3-[3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-

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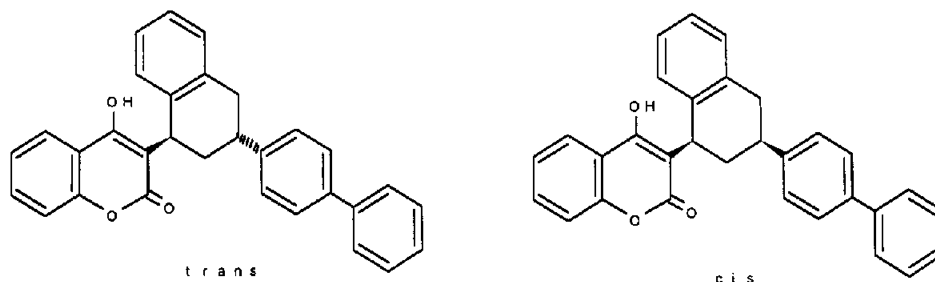

Fig. 1. Chemical structures of *trans*-difenacoum and *cis*-difenacoum.

1-naphthyl]-4-hydroxycoumarin) and flocoumafen (4-hydroxy-(3-[1,2,3,4-tetrahydro-3-[4-(4-trifluoromethylbenzyloxy)phenyl]-1-naphthyl]-coumarin)), a highly simplified method with minimal sample preparation has been developed for the extraction and HPLC analysis of the *cis* and *trans* diastereomers of difenacoum in rat liver and plasma. The diastereomers have been stereochemically assigned from their proton nuclear magnetic resonance spectra. Spectroscopic differences between the diastereomers in both ultraviolet absorption and fluorescence have also been identified. This method has been validated in both plasma and liver tissue and potentially could be used for the analysis and identification of other structurally related 4-hydroxycoumarin anticoagulants.

EXPERIMENTAL

Materials

All solvents were HPLC grade (Rathburn Chemicals Limited, Walkerburn, UK). Glacial acetic acid, polyethylene glycol, tri-sodium citrate, triethanolamine and ammonia solution (sp. gr. 0.88 g/ml) were obtained from BDH (Poole, UK). Warfarin, difenacoum (technical 91% pure, analytical 98% pure), brodifacoum (analytical 97% pure) and flocoumafen (analytical 96% pure) were kindly supplied by Sorex (Widnes, UK).

Separation of difenacoum diastereomers

The diastereomers were isolated from technical difenacoum by TLC using Anachem (Luton, UK) 2 mm GF 254 silica preparative TLC plates, developed with dichloromethane. The two fluorescent bands, corresponding to the diastereomers, were removed, and soxhlet extracted in dichloromethane for 4 h. The diastereomers were recrystallized from these extracts and purity was established by HPLC.

Mass spectrometry

Direct-insertion mass spectrometry, using a JEOL JMS DX 300 (Watchmead, UK) mass spectrometer operated at 70 eV in electron impact mode, was performed to confirm that the isolated materials were both difenacoum. Equivalent masses of the recrystallized diastereomers, were dissolved in acetonitrile and each sample (1 μl) loaded onto a glass insertion probe. A temperature gradient from ambient to 300°C, at 32°C/min, was used to volatilize the samples and the mass range scanned from m/z 20–500.

Nuclear magnetic resonance spectrometry

Samples of the two diastereomers (6.7 and 13.6 mg respectively) isolated from technical difenacoum were dissolved in 1 ml of deuterated chloroform (Cambrian Gases, Croydon, UK) and analysed by proton nuclear magnetic resonance spectrometry (NMR) on a Bruker AC 300 spectrometer (Coventry, UK) operating at 300.13 MHz for protons with a 20 degree pulse and a 3.4 s repetition time.

Ultraviolet and fluorescence spectrometry

Ultraviolet absorbance spectra of *cis* and *trans* difenacoum diastereomers (5.75 and 9.2 μg/ml re-

spectively) in acetonitrile were obtained using a Varian DMS 100 (Walton on Thames, UK) spectrophotometer, scanning from 500–190 nm at 20 nm/min with a band width of 0.2 nm. Emission spectra (315–550 nm) at an excitation wavelength of 310 nm were obtained using a Perkin-Elmer (Beaconsfield, UK) 3000 scanning fluorimeter. Fluorescence of the difenacoum diastereomers was also measured by excitation at 310 nm, bandwidth 2.5 nm, and emission at 390 nm, bandwidth 5 nm.

Chromatography

HPLC separation was carried out using a Waters (Watford, UK) system comprising three 510 pumps and a 710 WISP autosampler controlled by an 820 data station. Anticoagulants were detected using an LC 240 fluorimeter (Perkin-Elmer, Beaconsfield, UK), excitation and emission wavelengths were set at 310 nm and 390 nm respectively. A Waters Lambda Max 480 ultraviolet detector, set at 310 nm, was used as a second detector in series when the spectral characteristics of difenacoum were investigated. The reverse-phase analytical column (250 mm × 4.6 mm I.D.) was slurry packed with Zorbax ODS 5 μm (Hichrom, Reading, UK) using chloroform as the slurry medium and methanol as the packing medium.

The mobile phases, acetonitrile and water, were modified by addition of 0.1% (v/v) acetic acid [1], filtered, degassed and sparged with helium. A hyperbolic gradient (Waters type 2) was used to separate the diastereomers, using 55–75% acetonitrile over 20 min at a flow rate of 2 ml/min. The column was maintained at 35°C. A 5% (v/v) ammonia solution in water was added at 0.3 ml/min to the column effluent to ensure that the pH was >8 to maximise fluorescence of the anticoagulants [1]. Addition of methanol to the mobile phases at concentrations of up to 5% did not improve the separation.

Extraction of difenacoum from blood and liver of treated rats

Tolworth Albino Susceptible (TAS) rats, a warfarin-susceptible strain, were maintained as described previously [7]. Technical difenacoum was dissolved in polyethylene glycol 200–triethanolamine (9:1 v/v) to give a stock solution of 5 mg/ml and was administered to rats by intra-peritoneal injection, under light anaesthesia induced by diethyl ether, such that each animal received 5 mg/kg body weight of difenacoum. Blood samples (200 μl) were obtained from the caudal vein, under light ether anaesthesia, and immediately transferred into citrated tubes, vortex-mixed, and kept on ice. The citrated tubes were prepared in advance by pipetting 20 μl of a 3.13% (w/v) aqueous tri-sodium citrate solution, into 0.5 ml Eppendorf centrifuge tubes, then evaporating to dryness (37°C overnight). Citrated blood was centrifuged at 2000 × *g* for ten min. Aliquots (60 μl) from the resultant plasma samples were deproteinised [8] with 240 μl of acetonitrile in 300 μl autosampler insert vials, centrifuged at 2000 × *g* for 10 min to sediment the plasma proteins, and the vials loaded directly into the autosampler for HPLC analysis.

Livers were perfused *in situ* with ice-cold isotonic saline via the hepatic portal vein prior to removal. The tissue was homogenised, using an Ultra Turrax tissue disperser (Sartorius, Epsom, UK), in 15 ml of acetonitrile and centrifuged at 2000 *g* for 10 min. The pellet was resuspended and re-extracted by homogenisation in a further 10 ml of acetonitrile and the supernatants pooled, prior to HPLC analysis. In all cases 20 μl of the extracts were chromatographed. The recoveries from rat plasma and liver were determined by the addition of appropriate volumes of the stock solution to untreated samples prior to extraction, to give the concentrations described.

RESULTS AND DISCUSSION

Mass spectrometry of cis and trans difenacoum

Mass spectrometry confirmed that both diastereomers produced the expected mass ion at $M^+ = 444$, with similar fragmentation patterns. Two major fragments were produced from both diastereomers, corresponding to *m/z* 167, the phenyltropylium ion, and *m/z* 282 corresponding to the diphenyltetralin moiety remaining after de-

saturation and cleavage of the 4-hydroxycoumarin nucleus.

Assignment of stereochemical configurations from NMR data

Stereochemical assignment of the *cis* and *trans* configurations of 1,2-disubstituted tetrahydronaphthalene derivatives has been described [9]. It was assumed that the alicyclic ring was in the pseudo-chair form and the assignments were made according to the size of the coupling constant ($J_{1,2}$) between the protons on tetralin ring positions 1 and 2.

The conformational equilibrium of the 1,3-disubstituted tetralin ring in *cis*-difenacoum should strongly favour the *bis* equatorial conformer. Of the two protons on tetralin position 2, one would be located *trans* to the proton on position 1 and give a $J_{1,2}$ of approximately 10 Hz. The dihedral angle to the other would be much smaller and the other $J_{1,2}$ should be approximately 5 Hz. It is unclear whether the conformational equilibrium of *trans*-difenacoum will be dominated by the biphenyl or the 4-hydroxycoumarin substituent. If the biphenyl dominates and is therefore equatorial, then the 4-hydroxycoumarin substituent at position 1 must be axial and both $J_{1,2}$ will be approximately 5 Hz. If the 4-hydroxycoumarin dominates and is equatorial then one $J_{1,2}$ will be 10 Hz and the other much smaller, as with the *cis* isomer. If neither substituent dominates then rapid equilibration will occur and the two observed coupling constants will be the average of the individual conformers. It is apparent that the *cis* isomer must therefore include a $J_{1,2}$ of 10 Hz but this may not necessarily be the case for the *trans* isomer.

Fortunately, it is simple to identify unambiguously the NMR signal due to the proton on the tetralin ring position 1 since it is the only aliphatic proton in difenacoum which is doubly allylic and therefore occurs in a region of the spectrum which has no other signals. The diastereomer with the shorter retention time, under the HPLC conditions described above, gave a signal for this proton with a quadruplet of almost equal intensities centred at 4.9 ppm. The $J_{1,2}$ were 11.9 and 6.0 Hz and were interpreted as arising from the *cis* diastereomer. The diastereomer with the longer retention time by HPLC, produced a proton NMR spectrum with a triplet centred at 4.76 ppm, and the central component was approximately twice as intense as the others. The $J_{1,2}$ were both 4.20 Hz, and this was interpreted as being the *trans* diastereomer.

The diastereomers of the structurally related 4-hydroxycoumarin, flocoumafen, have been stereochemically assigned by X-ray crystallography of pure standards. Proton NMR spectroscopy of these standards demonstrated multiplets centred at 4.75 ppm for the *trans* diastereomer and 4.9 ppm for the *cis* diastereomer [10]. This evidence confirms that the stereochemical assignment of the two difenacoum diastereomers (Fig. 1), based on our interpretation of the NMR spectra, is correct.

Ultraviolet absorption and fluorescence spectral characteristics

Standards of these diastereomers as prepared by TLC, in acetonitrile, were chromatographed separately (Fig. 2). The peak area of the *trans* diastereomer, by fluorescence, was found to be 71% of the *cis* diastereomer, per unit mass. The fluorescence (Perkin Elmer 3000) of these same difenacoum standards at 390 nm (excitation at 310 nm) confirmed that the response of the *trans* diastereomer was less than that of the *cis* diastereomer. The areas under the curves of the emission spectra (315–550 nm) of these standards in acetonitrile indicated that emission from the *trans* diastereomer was 51% that of the *cis* diastereomer. Ultraviolet spectra of these same standards, in acetonitrile, demonstrated absorbance peak maxima for both diastereomers at 254 nm with a shoulder at 310 nm. Absorption at 310 nm of *cis*-difenacoum was 72% of the *trans* diastereomer per unit mass. Because of this inverse relationship between ultraviolet absorption at 310 nm and fluorescence when excited at the same wavelength, the responses of the two diastereomers were investigated under the same conditions used for the HPLC separation. A standard solution of the technical difenacoum (11 μg/

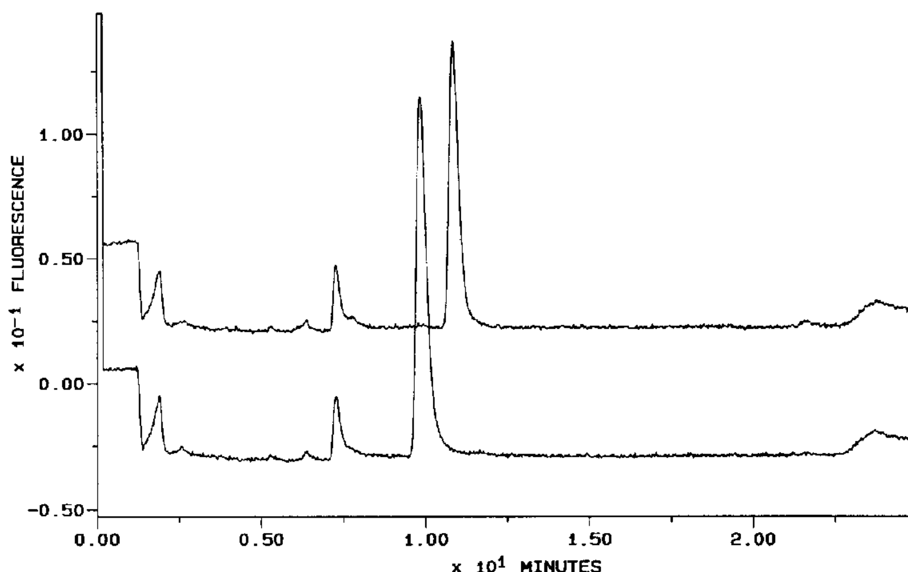

Fig. 2. Chromatograms of the diastereomers of difenacoum: *cis* 9.2 μg/ml (lower trace) and *trans* 11.5 μg/ml (upper trace). Fluorimeter attenuation 32, response factor 2, 20 μl injected.

ml) was chromatographed with both ultraviolet and fluorescence detection. The peak areas of the two diastereomers obtained by fluorescence were normalised for concentration by multiplying the peak area of the *cis* diastereomer by 0.71. The peak areas of the diastereomers obtained by ultraviolet absorption were normalised for concentration by multiplying the peak area of the *trans* diastereomer by 0.72. The *cis*:*trans* diastereomer ratios were thus calculated as 2.95 by fluorescence detection and 3.15 by ultraviolet absorption.

HPLC of difenacoum diastereomers

Under the chromatographic conditions described above, *cis* and *trans* diastereomers in a difenacoum mixture (analytical grade) eluted with retention times of 9.8 and 10.9 min respectively at a concentration of 74% acetonitrile. Peaks of the two diastereomers were ≥90% baseline resolved. Standard curves for the *cis* and *trans* diastereomers of difenacoum, based on peak area, were linear over the range 0.05–25 μg/ml in acetonitrile, typically with coefficients of determination (r^2) of 0.998 and 0.996 respectively. The minimum detectable amount of each isomer from standard solutions or plasma and liver extracts from treated animals, was 5 ng/ml at a signal-to-noise ratio of 3.

The potential of this method for the analysis of two closely related 4-hydroxycoumarin anticoagulants, brodifacoum and flocoumafen, was also examined. Standard solutions were prepared as described for difenacoum. The chromatograms (Fig. 3) show typical separations of diastereomers of the three compounds. Retention times and coefficients of determination of the standard curves are shown in Table I. Warfarin eluted as a single peak at approximately 4 min (data not shown).

Recovery of difenacoum added to plasma, water and liver samples

Six plasma samples from rats and six water samples were supplemented with the technical diasetereomeric difenacoum to give a final concentration of 1.37 μg/ml. Mean total recovery ± standard deviation was 1.38 ± 0.07 μg/ml from plasma and 1.36 ± 0.06 μg/ml from water, equivalent to recoveries of 101 ± 5% and 99 ± 4% respectively.

In preliminary pharmacokinetic experiments

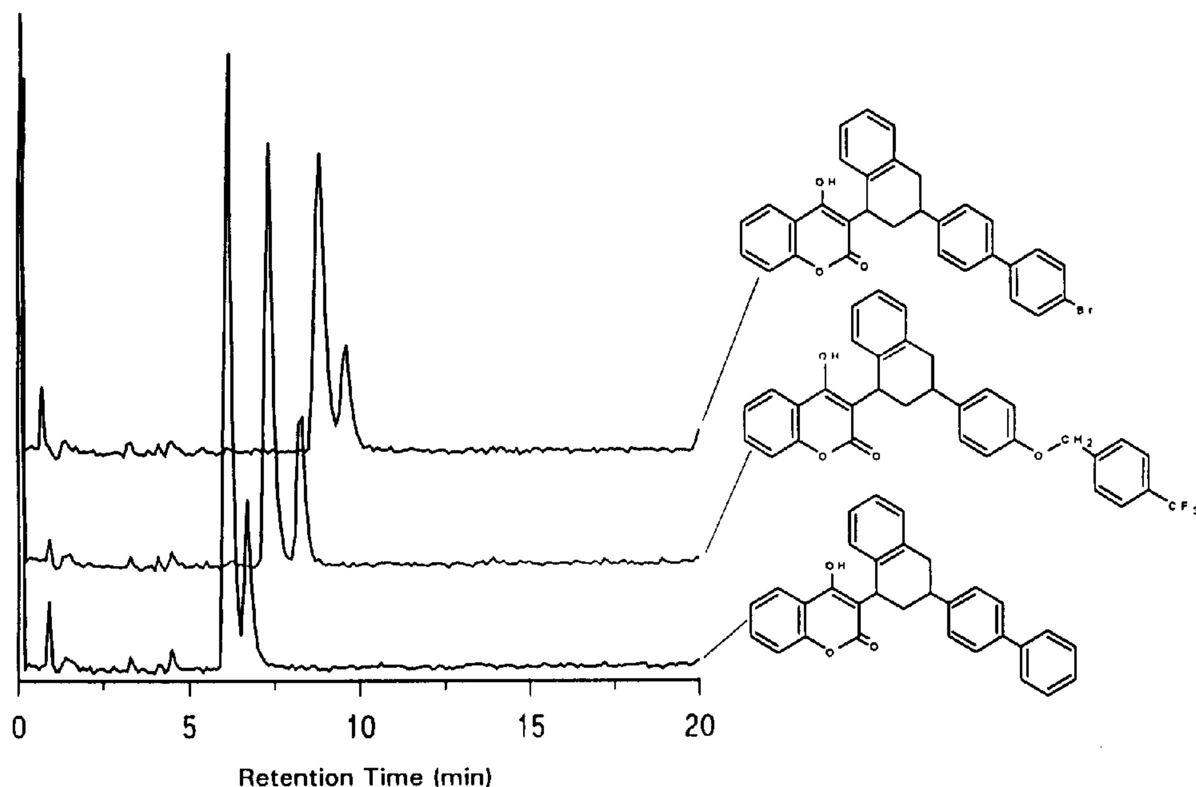

Fig. 3. Chromatograms of reference standards and chemical structures of difenacoum (605 ng/ml, lower) flocoumafen (600 ng/ml, middle) and brodifacoum (663 ng/ml, upper). Fluorimeter attenuation 16, response factor 2, 20 μl injected.

where rats were dosed by body weight (5 mg/kg), approximately 30% of the administered dose was found in the liver at peak plasma concentration which occurred at 5 h. Therefore, livers from five untreated rats were removed then supplemented, prior to extraction, with 300–400 μg of diastereomeric difenacoum, dependent on the body weight of the animal. Mean recovery was 92.6 ± 2.5%.

TABLE I

RETENTION TIMES AND COEFFICIENTS OF DETERMINATION (r^2) FROM STANDARD CURVES CONSTRUCTED FROM DUPLICATE ANALYSES OF STANDARDS IN THE RANGE 7.5–30 μg/ml, FOR *cis* AND *trans* DIASTEREOMERS OF DIFENACOUM, BRODIFACOUM AND FLOCOUMAFEN

Compound	Diastereomer	Retention time (min)	Coefficient of determination (r^2)
Difenacoum	*cis*	9.8	0.997
	trans	10.9	0.997
Flocoumafen	*cis*[a]	11.9	0.995
	trans	13.5	0.997
Brodifacoum	*cis*[a]	14.3	0.994
	trans	15.6	0.998

[a] Assigned on the basis of retention times by HPLC.

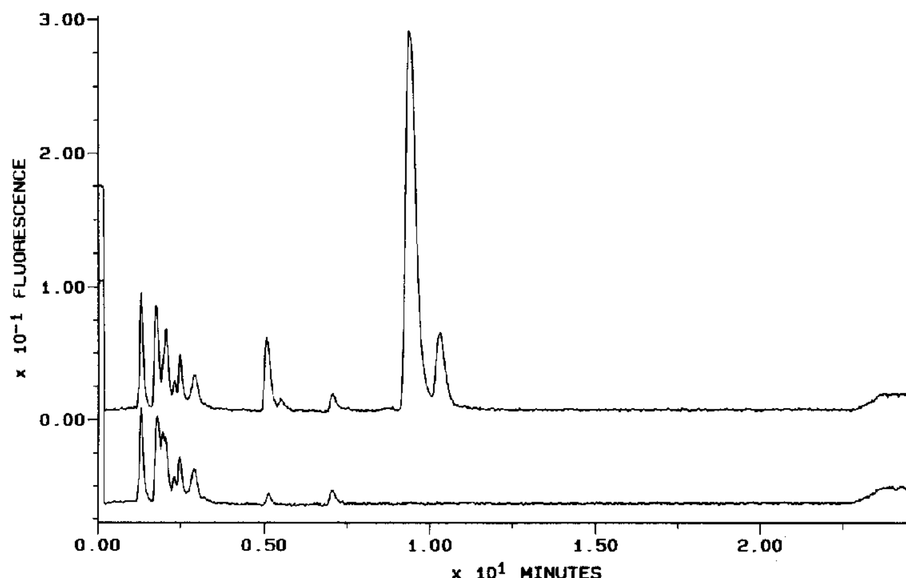

Fig. 4.Chromatograms of *cis* and *trans* diastereomers of difenacoum following extraction from rat plasma 5 h after treatment with 5 mg/kg difenacoum (upper trace), and plasma from a sham-treated animal which received vehicle alone (lower trace). Fluorimeter attenuation 32, response factor 2, 20 μl injected.

Chromatography of extracts of blood and liver from difenacoum-treated rats

A typical chromatogram of difenacoum in plasma, 5 h after intra-peritoneal administration, is shown in Fig. 4. Chromatograms of the liver extracts from treated rats were similar to those obtained from plasma samples. Blank plasma and liver extracts from sham-treated rats were both free from interfering coextractives. Blank extracts supplemented with difenacoum did not result in changes in the chromatography or alter the quantitation of the diastereomers compared to standard solutions.

CONCLUSION

This method provides a simple, rapid and sensitive technique for the analysis of diastereomers of difenacoum in blood or liver tissues. It should be recognised that the method of detection can have pronounced effects on the relative peak areas of the diastereomers, which could result in systematic errors in quantitation. Additionally, this method shows promise for the quantitative analysis of the diastereomers of brodifacoum and flocoumafen. However, it should be noted that the extraction procedure has not yet been validated for the latter two anticoagulants nor have the diastereomers of brodifacoum been positively identified. Because of its simplicity this technique has been applied [11] to investigate unexplained hypothrombinaemic episodes encountered clinically and is being used for studies of the pharmacokinetics of difenacoum diastereomers in the rat. The method could readily be applied to investigations of the environmental and toxicological impact of these second-generation anticoagulants on non-target species of mammals and birds.

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