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ELECTRON CAPTURE AND MULTIPLE ION DETECTION OF BENZODIAZEPINE ESTERS IN PHARMACOKINETIC STUDIES

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SUMMARY

A sensitive and highly specific gas–liquid chromatographic method is described for the determination of benzodiazepine esters. The method involves the extraction of pivoxazepam and 2′-chloropivoxazepam from blood and urine samples and the hydrolysis of both drugs with strong acid to the corresponding 2-amino-5-chlorobenzophenone (ACB) and 2-amino-5,2′-dichlorobenzophenone (ACDB). Recoveries of 97–98% have been obtained and the trifluoroacetic (TFA) derivatives of both benzophenones (ACB-TFA and ACDB-TFA) can be chromatographed on OV-17, in a short time, with good electron-capture detector (ECD) responses. The mass spectra of these derivatives provide abundant molecular ions suitable for mass spectrometric detection by multiple ion detection (MID), with a considerable improvement in specificity compared with the ECD responses. The detection limits in blood specimens have been established at 5–10 ng/ml by ECD and 0.5–0.8 ng/ml by MID. The method has been applied to the study of blood levels attained after i.v. and oral doses in rats and in man.

INTRODUCTION

1,4-Benzodiazepines are widely used as psychotropic drugs[1] and constitute a relatively new class of compounds that includes various chemically related derivatives, such as diazepam, oxazepam, chlordiazepoxide and flurazepam. A recent addition to this series, 7-chloro-1,3-dihydro-5-phenyl-1H-2-oxo-3-pivalyloxy-1,4-benzodiazepine[2] or pivoxazepam[3], exerts an anxiolytic activity that prevails over other pharmacological effects. In relation to its parent drug, oxazepam, the former drug has weaker sedation, hypnosis and muscle relaxation effects, which suggest that it may be a "pro-drug" with good potential for clinical applications[4]. The main metabolic pathway of pivoxazepam proceeds according to the scheme[5] given in Fig. 1.

The pharmacokinetic study of this drug requires an analytical method suitable for the quantitative determination of the intact drug at nanogram levels, and at the same time specific enough to differentiate the drug from its own metabolites and/or

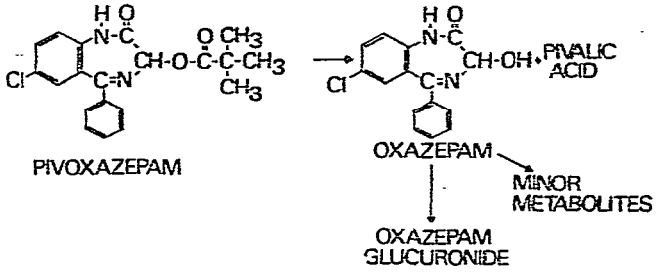

Fig. 1. Metabolic pathway of pivoxazepam.

other interfering components in physiological fluids. In this sense, the experiments carried out have shown that the necessary sensivity levels can be attained by gas–liquid chromatography (GLC) with electron capture detection (ECD), as also described by other workers[6,7].

However, in our experience, the ECD does not provide sufficient specificity of response to cope with the usual complexity of biological extracts, a fact also verified in the course of this work. For this reason, seeking higher sensitivities and greater specificity, we investigated selected ion monitoring techniques[8], designing and building our own alternating voltage accelerating unit for multiple ion detection (MID) purposes[9], a brief account of which is also included here.

To our knowledge, GLC–MID has not been used previously for the biological assay of benzodiazepines, which is surprising in view of the lower limit of detection obtained for pivoxazepam (500–800 pg/ml in plasma compared with 5–10 ng/ml with the ECD) and the high level of specificity observed.

The method developed involves the detection of pivoxazepam as the corresponding trifluoroacetylated benzophenone using a chlorinated analogue as the internal standard. The method has been applied to the assay of human and rat plasma levels and also to studies related to the urinary elimination of the drug in rats. It has also been shown that the method described can be applied to other benzodiazepine pivalates, presently under study in our laboratories.

EXPERIMENTAL

Chemicals and reagents

The chemicals used for calibration and testing purposes were 2-amino-5-chlorobenzophenone (ACB), pivoxazepam, lorazepam pivalate (2′-chloropivoxazepam) and 2-amino-5,2′-dichlorobenzophenone (ADCB) (Synthesis Department, Ferrer Internacional, Barcelona, Spain).

The reagents used were trifluoroacetic anhydride (TFAA) (Merck, Darmstadt, G.F.R.) and hexamethyldisilazane (HMDS) (Pierce Eurochemie, Rotterdam, The Netherlands). The solvents used were pyridine, 0.05 *M* in benzene; 99% *n*-hexane (Merck), washed with concentrated sulphuric acid; diethyl ether (Carlo Erba, Milan, Italy), RP grade, free of peroxides; benzene (Merck); and acetone (Carlo Erba), RP grade, kept over anhydrous sodium sulphate. The solvents were redistilled before use. The buffers and neutralizing solutions used were 1 *M* phosphate buffer (pH 7.4), 1 *M*

acetate buffer (pH 4.5), 6 *N* sodium hydroxide solution and 6 *N* hydrochloric acid. The water used in extractions and the preparation of buffer solutions was thrice-distilled.

Extraction procedure for pivoxazepam

A 3-ml volume of heparinized plasma is extracted with 9 ml of benzene containing an appropriate amount of 2′-chloropivoxazepam (internal standard). A portion of the benzene phase (4 ml) is then extracted three times with 5 ml of 1 *N* hydrochloric acid, 3.5 ml of the benzene phase remaining after the extractions are taken to dryness and the residue is redissolved in 40 μl of absolute ethanol, adding 5 ml of 6 *N* hydrochloric acid and keeping the solution at 100° for 1 h. This acidified solution is neutralized with sodium hydroxide solution, using 40 μl of a 1% solution of bromothymol blue as indicator.

The solution containing the benzophenone thus formed is adjusted to pH 7.4 with 1 ml of phosphate buffer and extracted twice with 10-ml volumes of diethyl ether, subsequently adding the necessary amount of solvent to give a final volume of 25 ml.

Derivatization

The ether extracts (20 ml) are taken to dryness and the residue is then treated with 10 μl of TFAA and 25 μl of a 0.05% solution of pyridine in benzene, maintaining the mixture at 60° in a tightly closed vial for 10 min. After careful elimination of the solvent and reagent, the residue is redissolved in 100 μl of *n*-hexane–acetone (1:1), injecting 2 μl of the solution obtained into the gas chromatograph.

Note that before attempting to derivatize the sample it is necessary for it to be free of any traces of water. For this purpose, the ether extracts are concentrated to 1 ml in a rotary film evaporator and centrifuged. If any appreciable amount of water remains, it is removed with a clean syringe and the ether is evaporated under a stream of purified nitrogen. After keeping the residue in a desiccator for 1 h, it is ready for derivatization as indicated above.

Gas chromatography

The separations were carried out on a Perkin-Elmer Model 900 gas chromatograph equipped with a nickel-63 ECD. The glass columns (2 m × 2 mm I.D.) were treated with a 10% solution of HMDS in *n*-hexane, dried and packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.).

The columns were conditioned for 16 h at 300° with nitrogen at a flow-rate of 30 ml/min. Argon–methane (95:5) was used as the carrier gas, adjusting the flow through the ECD to a total of 160 ml/min by means of the auxiliary carrier gas accesory. The detector was operated for maximum sensitivity at a 50 μsec pulse interval, and the column temperature was set at 220°.

Gas chromatography–mass spectrometry (GC–MS)

The mass spectra of the various compounds and derivatives were obtained with a single-focusing Hitachi RMU-6H mass spectrometer, coupled through a new type of single-stage jet interface to a Perkin-Elmer Model 3920 gas chromatograph. The operating conditions of the mass spectrometer were as follows: electron energy,

70 eV; emission current, 80 μA; accelerating voltage, 2400 V; ion chamber temperature, 200°; separator temperature, 220–240°.

A novel feature of the GC–MS interface is that all of the transfer lines from the column exit leading to the jet unit and from there on to the mass spectrometer inlet are made of 1/16-in. O.D. stainless-steel tubing containing an inner gold capillary. The unit is manufactured for Hitachi by Perkin-Elmer (Norwalk, Conn., U.S.A.).

Multiple ion detection

As the Hitachi mass spectrometer is not equipped with the necessary alternating voltage facilities for single ion detection or MID of selected components in a gas chromatographic profile[10,11], we undertook the design and construction of a four-channel MID unit suitable to our specifications. The design and operation of this unit, which will be reported in detail elsewhere[9], can be briefly summarized as follows. The system consists of six main components: (A) a programmed 0–1100 V voltage source; (B) a digital programmer; (C) a variable ramp generator; (D) a signal distributor; (E) four holding amplifiers; and (F) an oscilloscopic monitor.

In this system, a regulated voltage increment given as a function of time, $\Delta V(t)$, is added to the nominal accelerating voltage (V) in each of the four channels. This $\Delta V(t)$ is the sum of a fixed ΔV supplied by the programmed voltage source (A) and a variable voltage sawtooth wave which is supplied by the ramp generator (C), thus obtaining a "miniscan" around each of the selected masses. The addition of the four different $\Delta V(t)$ values and their synchronization with the horizontal scan of the oscilloscopic monitor (F) is driven by the digital programmer (B). The regulated voltage (ΔV) can be changed continuously from 0 to 1100 V, which allows the operator to cover a maximum mass range of 30% of the lowest range mass at $V = 3600$ V and of about 90% at $V = 1200$ V.

The four signals are collected separately by means of a signal distributor (D), which is also synchronized with the voltage source (A) and the horizontal scan of the monitor (F). These signals are amplified through four holding amplifiers (peak detector and S/H amplifier). The oscilloscopic monitor is used for the initial search and focusing operations, which are based on the background reference peaks, owing to the lack of a mass marker, and provides likewise a real time control for the correct focusing of each peak. These features contribute to the minimization of the errors due to defocusing effects[12], allowing the addition of ΔV's as high as those described. The miniscan time is continuously adjustable between 0.5 and 12 sec and the switching time between two adjacent channels is 50 msec. These relatively high scan times permit the use of high time constant filters, with the consequent decrease in noise and therefore increase in sensitivity, expressed as signal-to-noise ratio.

Pharmacokinetic studies

Experimental animals. This work was carried out using male SPFF rats weighing on average 236.8 g. The animals were subjected to i.v. (2.58 mg/kg) and oral (30 mg/kg) doses of pivoxazepam, equivalent to 2 and 23.2 mg, respectively, of free oxazepam per kilogram of body weight.

Blood extractions after i.v. injections were performed at 5, 10, 20, 30 and 40 min and 1, 2, 3 and 5½ h. The first two points of the dose–response curves were ob-

tained with ten animals and the remainder with five. The responses to oral administrations from 0 to 24 h were followed, drawing blood samples at each hour. For the assay of 24-h urine samples, the subjects received doses of 50 mg per kilogram of body weight.

Humans. Four healthy volunteers each received one capsule containing 25.86 mg of pivoxazepam, equivalent to 20 mg of free oxazepam. Blood samples were drawn before the first administration and after 1, 2, 4, 6, 8, 12 and 24 h.

RESULTS AND DISCUSSION

Gas chromatographic separations

The initial attempts to chromatograph pivoxazepam on OV-17 were unsuccessful, as it apparently undergoes an extensive pyrolytic breakdown, either on the injection port or on the column itself, as indicated by the complexity of the 2-h GLC multiple peak pattern obtained. However, silanization of both pivoxazepam and the internal standard, 2'-chloropivoxazepam, with a BSA–1 % TMCS mixture in acetonitrile gave sharp symmetrical peaks with retention times of the order of 8 and 10 min, respectively, at 270°. Nevertheless, the quantitative response of the ECD to the TMS derivatives did not meet our specifications in terms of the necessary sensitivity levels required for biological applications (<50 ng/ml in urine or plasma).

This aspect is reflected in Fig. 2A, which shows the GLC profile obtained by extraction of a urine sample according to the procedure outlined, which included the acid hydrolysis of pivoxazepam to yield the corresponding 2-amino-5-chlorobenzophenone (ACB). The retention time of ACB is of the order of 20 min and the peak tails considerably. In contrast, the TFA derivative of ACB produced a sharp peak (Fig. 2B) with a retention time of about 6 min, much like the TMS derivatives of pivoxazepam but with a significantly higher response on the ECD.

Under these conditions, the profile of the urine extract (Fig. 2B) appears cleaner and the peak presumably corresponding by retention time to the acylated ACB of pivoxazepam is more amenable to the quantitative determination of low dose–response curves in comparison with the corresponding ACB peak in the upper trace of Fig. 2A.

Hydrolysis and extraction of pivoxazepam

In concentrated acid, the molecule of pivoxazepam breaks down, yielding the same aminochlorobenzophenone as that obtained from oxazepam[13]. On the other had, the possibility of transforming pivoxazepam into oxazepam by basic hydrolysis has been proved unfeasible because the molecule decomposes to various by-products without forming the expected benzodiazepin.

The determination of pivoxazepam as 2-amino-5-chlorobenzophenone, which, as stated, could also be derived from oxazepam, obviously requires the separation of both compounds, precursor and metabolite, prior to the hydrolysis in concentrated hydrochloric acid.

Pivoxazepam and its free metabolites, as well as the internal standard used for its determination (2'-chloropivoxazepam), can be efficiently extracted with benzene, as described for diazepam[14]. On the other hand, the separation of the unconjugated metabolites of pivoxazepam, including oxazepam, can be accomplished quantitatively

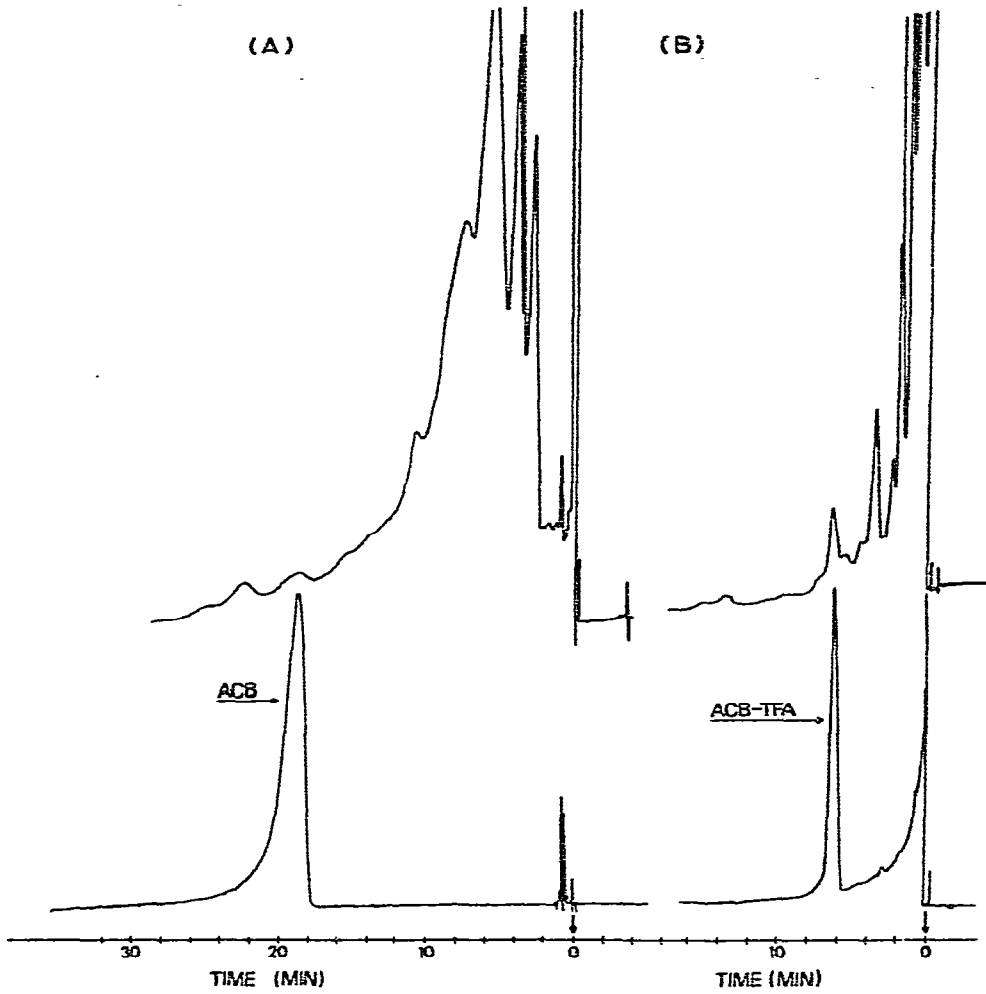

Fig. 2. (A) Gas chromatogram of the free 2-amino-5-chlorobenzophenone (lower trace) and chromatographic profile obtained after the acid hydrolysis of an extract of a urine pool from various rats administered an oral dose of 50 mg/kg of pivoxazepam (upper trace). (B) Same samples after derivatization of both with TFAA. Chromatographic conditions for (A) and (B): 2 m × 2 mm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh; oven temperature, 200; injector and detector blocks, 270°; carrier gas, Ar–CH_4 at a flow-rate of 30 ml/min.

by extraction of the benzene phase with 1 *N* hydrochloric acid, which prevents the possible premature degradation of pivoxazepam before its hydrolysis to the benzophenone with 6 *N* hydrochloric acid.

From the point of view of the extraction procedure, the method described is highly specific in the sense that the partition coefficient of pivoxazepam (K = [organic phase]/[aqueous phase]) favours the organic phase, with over 99% of pivoxazepam remaining in the benzene phase. A K value of 0.45 has been reported for the extraction of oxazepam from a diethyl ether phase with 1 *N* hydrochloric

acid[15], while the corresponding value in 1 *N* hydrochloric acid in benzene is 0.17, and even in 6 *N* hydrochloric acid the value is only 0.35.

Thus, the separation of the intact drug and its metabolites can be easily accomplished by re-extraction of the initial benzene phase with dilute hydrochloric acid owing to the low partition coefficient of oxazepam in this system.

Recoveries

The calibration graph obtained by adding various amounts of pivoxazepam and 2′-chloropivoxazepam, extracting and analyzing in the manner described, indicates that the recoveries change depending on the weight ratio of sample to internal standard. A maximum recovery of 97.7% for a series of five replicate determinations was obtained when the weight ratio of pivoxazepam to 2′-chloropivoxazepam was 0.65 (the respective peak heights corresponding to this weight ratio give a ratio close to unity.

Concurrently with the analysis of biological specimens, it is advisable to prepare a calibration graph by adding known amounts of pivoxazepam plus internal standard to equivalent volumes of blank specimens. The amounts added must be in the same range as that expected for the sample. This is needed in order to determine, from each weight ratio given by the calibration graph, the recovery factor that must be applied to obtain the initial amount of pivoxazepam present in the sample.

GLC calibration and quantitative responses

Although none of the reports related to the analysis of benzodiazepines mentions the determination of their benzophenone by-products as acyl derivatives, we have verified their considerable advantages with regard to (1) their lower retention times (Fig. 2B), which allow the use of lower oven temperatures, (2) less peak tailing and (3) higher sensitivity with the ECD.

For equivalent amounts of both compounds, the response of the TFA derivatives of ACB is about 20 times higher than that of the free ACB. The upper trace in Fig. 3 shows the response curve for ACB-TFA using ADCB-TFA as the internal standard. The linear regression coefficient for the data shown has been estimated as 0.983, with a standard deviation varying between ± 1.2 and 2.5% in an R_A range of 0.5–1.5 (where R_A is defined as the area ratio of the peak due to ACB-TFA to that of ADCB-TFA). For this reason, the internal standard is always added in an amount adjusted so that the value of R_A falls within this range. The linearity of response is limited to a power of ten range (linear between 0 and 0.1 ng and between 0.1 and 1 ng), which requires the adjustment of calibration graphs to the concentration range of the sample. The minimum absolute amount of ACB-TFA detected is lower than 25 pg at an attenuation of 40.

However, in plasma extracts, the limit of detection of ACB-TFA has been established as 2–4 ng/ml (corresponding to 5–10 ng/ml of pivoxazepam). These values were determined with a confidence limit of $\pm 10\%$ (defined as the value of the standard error times the estimated Student t value) for ten replicate runs, assuming an optimal weight ratio (R_w in Fig. 3). The results are statistically significant at the 95% confidence level ($P < 0.05$). The magnitude of the variations in the limit of detection observed as the method is applied to physiological fluids seem to be strongly dependent on the presence and relative levels of interfering substances, as shown by the blank

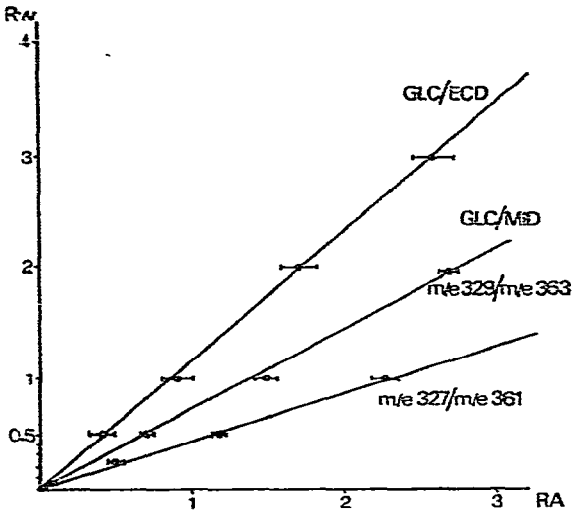

Fig. 3. GLC–ECD and GLC–MID quantitative response curves. ECD curve: Rw is defined as the value of the ACB-TFA:ADCB-TFA weight ratio. RA defines the corresponding area ratios obtained from the chromatograms. MID curves: Rw is the same weight ratio. RA gives the values of the peak height ratios of the ion current profiles of the peaks at *m/e* 329/363 and 327/361.

controls. This problem is considerably reduced by the use of selected ion monitoring techniques for multiple ion detection, as described below.

Multiple ion detection

In view of the good results recently obtained in the laboratory by single ion monitoring[10,11] or multiple ion detection (MID)[16], we decided to apply the latter technique to these benzodiazepine esters as a means of achieving greater specificity and, if possible, a lower limit of detection.

The first step consisted in the evaluation of the mass spectra of these substances, which are shown in Fig. 4. The mass spectrum of the free benzophenone shows a major ion at $M - 1$ (*m/e* 230), which in principle would be suitable for MID[17]. However, in biological determinations in general it is more useful to focus on higher mass values, in order to avoid the larger relative contribution of stationary phase bleeding and/or interfering components to the lower end of the mass spectral region. In this sense, the TFA derivative adds to its inherent GLC advantages a relatively high abundance molecular ion at *m/e* 327 (70%). Likewise, the internal standard also shows a relatively high peak at *m/e* 361 (85%). These ions plus their corresponding ^{37}Cl isotope peaks at *m/e* 329 and 363, respectively, where the peaks selected for MID. Their ion current profiles, obtained with the four-channel monitoring system, are also shown on the right-hand side of Fig. 4. Once these ions are selected and the mass spectrometer operating parameters are properly adjusted for their detection, a response is obtained only when a compound giving rise to any of these four ions is eluted from the chromatograph.

As seen in these profiles, the peaks obtained at about 7 and 9 min are in accordance with the expected retention times of the TFA derivatives of ACB and

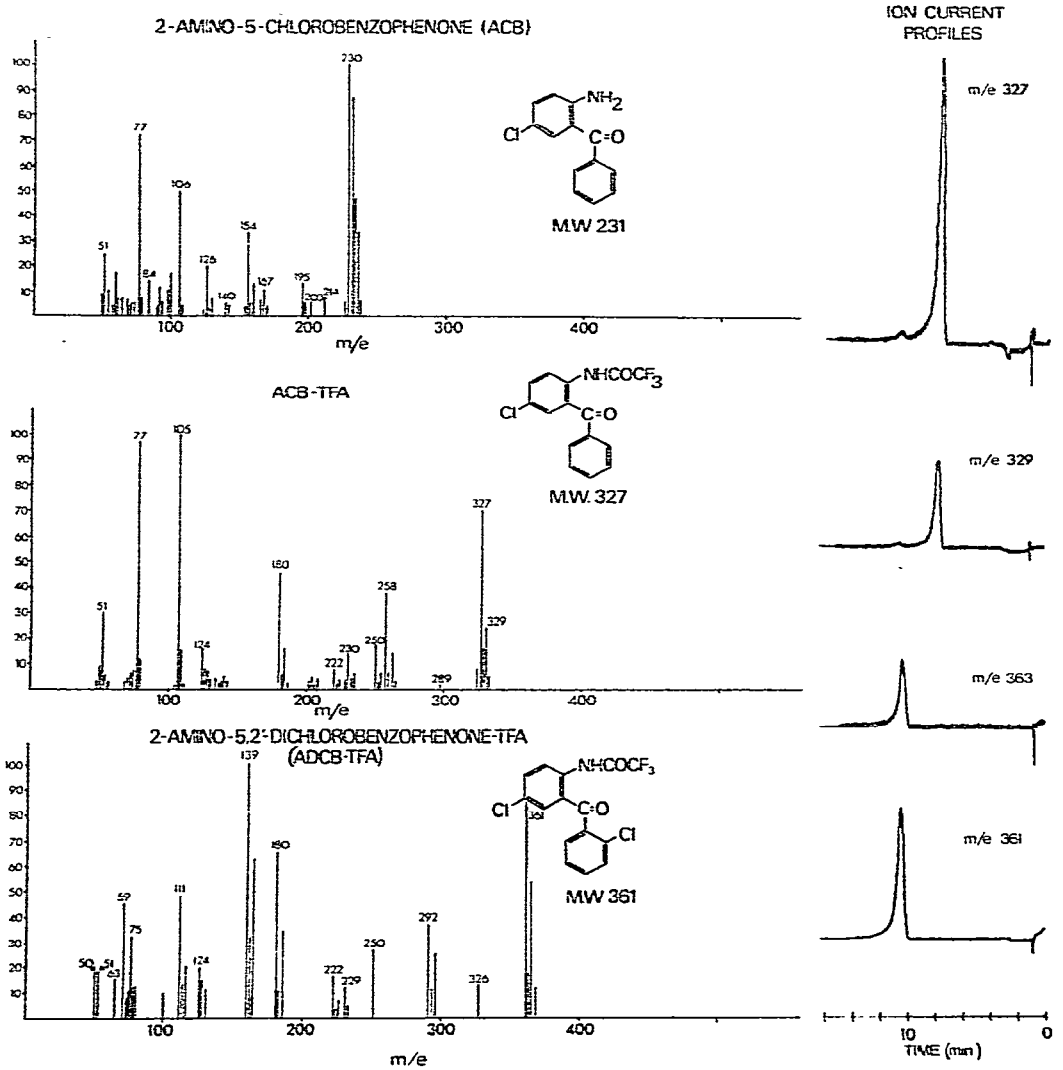

Fig. 4. Mass spectra of the free benzophenone and TFA derivatives of ACB and ADCB. The ion current profiles shown on the right illustrate the responses obtained by focusing the four recording channels of the MID on ions *m/e* 327 and 329 for ACB and *m/e* 361 and 363 for ADCB. These profiles were obtained under the following conditions: ionization energy, 70 eV; emission current, 80 μA; nominal acceleration voltage, 2400 V for *m/e* 363 + the corresponding ΔV values required to focus on the other three ions at a constant magnet current of 260 mA; source temperature, 200°; interface, 250°. Chromatographic conditions as indicated for Fig. 2, except for the column temperature, which was set at 220°.

ADCB. Note that these ion pairs have also been selected because of their characteristic abundance ratios. The *m/e* 327:329 ratio is *ca.* 3 for the monochlorinated benzophenone and *ca.* 1.5 for the dichlorinated analogue (ratio *m/e* 361:363). This is a very specific feature that carries significant weight with regard to the unequivocal identification of these substances in biological extracts. The considerable specificity of

detection thus achieved is reflected in the practical example in Fig. 5, which compares the results obtained with the ECD and those obtained by MID. The left-hand traces depict the GLC profile of an extract of human plasma obtained 2 h after ingestion of an oral dose of pivoxazepam. It is obvious that the ECD response of the ACB-TFA and ADCB-TFA peaks may be affected by the levels of other interfering components that are either co-eluted exactly with them or are eluted with a similar retention time. The retention times of the two benzophenone derivatives are indicated by the arrows on the GLC pattern of a plasma blank. On the right-hand side are shown the ion current profiles of the two ion pairs selected. The specificity is absolute in this instance. The peak of the ACB-TFA is unequivocally identified by (1) its responses at *m/e* 327 and 329, (2) their relative abundance ratio and (3) the retention time. The same reasoning applies to the ADCB-TFA.

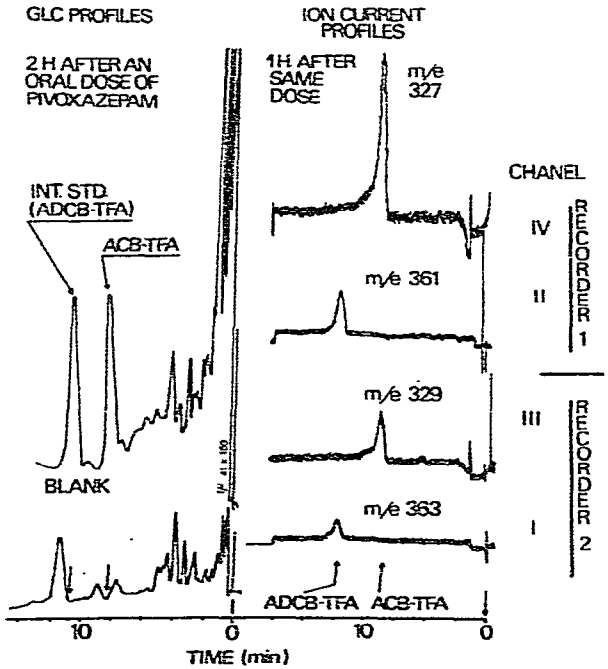

Fig. 5. Gas chromatographic profiles of an extract of human blood drawn 2 h after an oral dose of pivoxazepam. The blank shown below corresponds to the extract of a blood sample taken before the administration of the drug. On the right are shown the ion profiles of the masses selected for identification of the two derivatives. One of the pen recorders registers the two molecular ions *m/e* 327 and 361 (see Fig. 4) focused on the MID channels IV and II, respectively, while the corresponding $M + 2$ ions on channels III and I are registered on a second recorder. The peak height ratios thus obtained are used to construct the calibration graphs (Fig. 3).

It is worth noting that the results far exceeded our expectations with regard to the extreme degree of specificity achieved as it would not have been unreasonable to see the appearance of some extra peaks derived from other components of the plasma extract that could possibly also contribute to these mass values. However, when extra peaks appear they do so at different retention times and their relative abundances do not agree with the calculated ratios[11].

MID response curves

Fig. 3 also shows the GLC–MID calibration graphs compared with the ECD response in the same range of area and weight ratios. To obtain these graphs, the four signals coming from the signal distributor of the MID unit were fed to two dual-pen recorders so that one of the recorders received the ion currents generated by the molecular ions of the two benzophenones (*m/e* 327 and 361) and the other recorder received the signals generated by their respective isotope $M + 2$ peaks (*m/e* 329 and 363). For instance, this arrangement is indicated in Fig. 5.

Although not shown in Fig. 3, the response is linear for values of R_A in the range 0–8, equivalent to R_w values of 0–4, and the dispersions observed for replicate measurements are 40–50% lower than by GLC–ECD.

The absolute lower limit of detection by MID has been established at 300 pg for ACB-TFA, and for biological assays the limit is 500–800 pg/ml.

The heptafluoroacylated derivatives of ACB and ADCB were also tried initially but, although their ECD response was twice that of the TFA derivatives and their GLC behaviour was as good, their mass spectral pattern did not provide sufficiently abundant high-mass ions for MID. For instance, there are no peaks higher than 30% past the base peak at *m/e* 105 (the ion at *m/e* 258 shows a relative abundance of 30% while the molecular ion at *m/e* 427 represents only 12% of the base peak). However, the *m/e* 427 and 429 ions could still prove useful in the event that any interference appearing at the masses selected for the TFA derivatives would make it necessary to focus on higher masses in order to improve specificity.

Pharmacokinetic studies

Fig. 6 shows the blood fall-off curve of pivoxazepam in rats after an i.v. dose (2.58 mg/kg). The various points were obtained taking serial blood samples at the times indicated (0–5½ h). Details and statistical data are given in Table I. Control

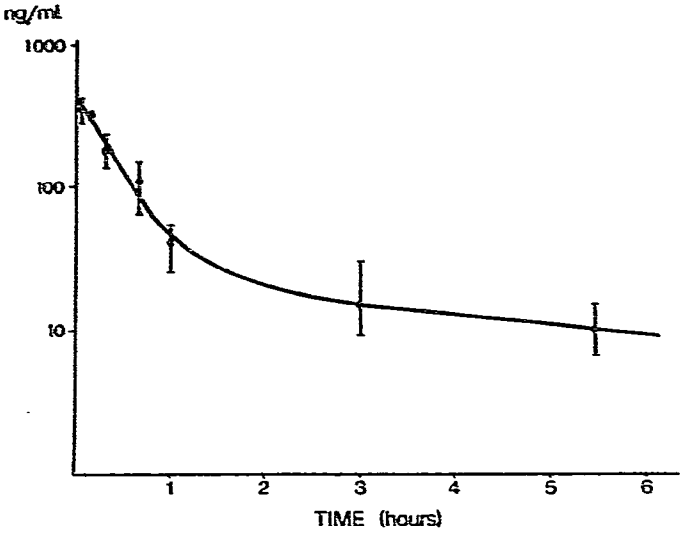

Fig. 6. Blood level fall-off curve after intravenous administration of a pivoxazepam dose (2.58 mg/kg) in rat.

TABLE I

BLOOD PLASMA CONCENTRATIONS (ng/ml) OF PIVOXAZEPAM IN TWO SERIES OF 5 AND RATS FOLLOWING ADMINISTRATION OF 2.58-mg/kg DOSES

Values at 5 and 10 min were obtained with 10 rats; the remainder of the entries apply to 5 rats. S.D. = standard d viation; S.E. × t = confidence interval. As the size of the populations sampled differs, these values are only cor parable within each of the two populations; S.D./$\bar{x}$ represents the corresponding coefficient of variation th allows a direct comparison among the values obtained at various times.

Time (min)	*Rat No.*										*Mean* ($\bar{x}$)	*S.D.*	*S.E.*×t (%)	*S.D.*/$\bar{x}$
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*				
5	311.7	234.9	437.7	312.1	507.2	248.8	263.0	490.5	604.4	220.4	363.0	60	± 23.6	0.165
10	350.1	339.8	316.0	294.6	—	341.2	382.2	333.0	333.0	305.3	332.8	12	± 5.9	0.036
20	131.5	147.8	165.8	233.3	206.2	—	—	—	—	—	176.9	19	± 29.5	0.107
40	138.6	130.2	138.6	76.9	79.3	—	—	—	—	—	112.7	14	± 34.9	0.124
60	17.7	82.9	34.2	43.7	29.4	—	—	—	—	—	41.6	11	± 74.2	0.264
180	—	37.3	21.3	63.4	13.3	—	—	—	—	—	15.6	10	±103.7	0.641
330	16.5	8.0	13.3	5.8	6.1	—	—	—	—	—	9.9	2.2	± 59.0	0.222

specimens were drawn before the administration of the first dose. The drug is detected at its maximum concentration at 5 min, declining progressively with time.

The relatively high blood levels of the intact drug attained after an i.v. dose facilitate the study of its elimination pattern by GLC–ECD. However, the lower levels detected after oral doses underline the relative importance of the presence of any ECD interfering substances in the control blanks (Fig. 5), which is a contributory factor to the sample variations observed. These are the type of samples where the GLC–MID method has proved most useful for its specificity.

Fig. 7 shows the blood levels observed in rats in response to an oral dose of pivoxazepam (30 mg/kg) throughout a 24-h period. The points corresponding to the upper trace were determined by GLC–ECD while the lower trace was obtained by MID. These curves are representative of the type of results that we have consistently obtained with both methods.

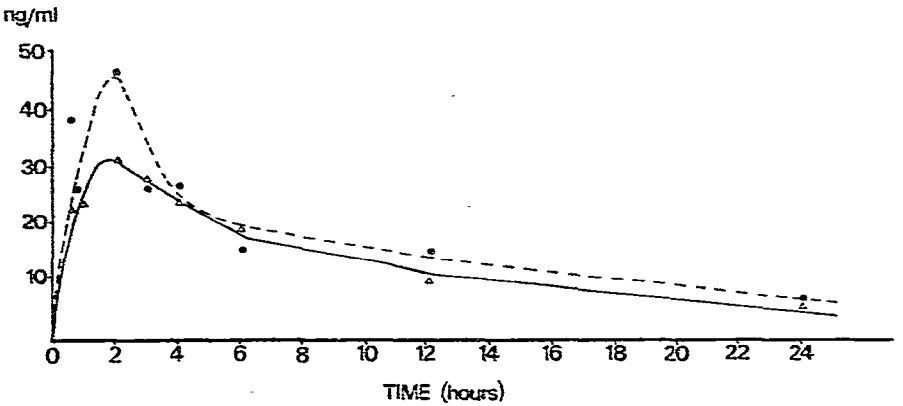

Fig. 7. Blood level fall-off curves following the oral administration of a dose of pivoxazepam in rat (30 mg/kg). ●, Curve obtained by GLC–ECD analysis of the extracts; △, curve obtained by GLC–MID analysis of the same samples.

Likewise, as the internal standard itself shows interesting pharmacological properties, its own elimination profile has been studied in a similar way, using pivoxazepam as the internal standard. The data obtained prove the feasibility of the GLC–MID method described for the determination of other benzodiazepine ester analogues.

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