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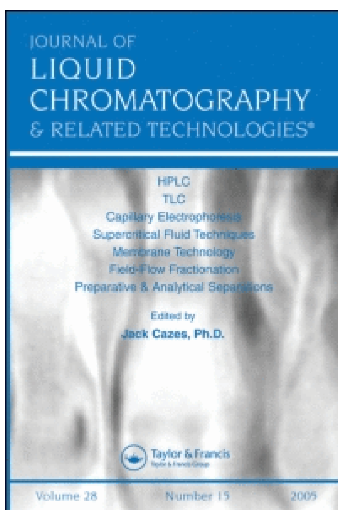

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A Sensitive High Performance Liquid Chromatographic Method for U-80, 278A, A Substituted Aminotetralin, in Rat Plasma, Whole Blood, and Brain Tissue

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A SENSITIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR U-80,278A, A SUBSTITUTED AMINOTETRALIN, IN RAT PLASMA, WHOLE BLOOD, AND BRAIN TISSUE

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ABSTRACT

A sensitive analytical method for U-80,278A, a substituted aminotetralin analogue in rat plasma, whole blood and brain tissue has been developed. The method involves solid phase extraction, efficient reversed phase HPLC and fluorescence detection, and can measure 1 ng/ml from 50 µl samples. During method development, many analogues were investigated and a wide range of extraction and HPLC conditions were explored. These experiments enabled rapid modification and revalidation of the method to support animal experiments with novel analogues.

INTRODUCTION

A series of substituted aminotetralins has been investigated as novel drugs showing CNS activity [1]. Routine bioanalytical assays for a number of analogues in a variety of biological matrices were required to

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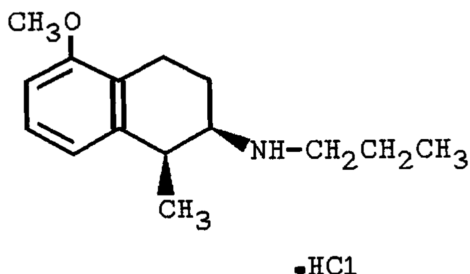

Figure 1

Structure of U-80,278A

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support bioavailability and other pharmacokinetic studies in the rat.

U-80,278A (fig. 1) was one of the first compounds to be investigated *in vivo*. Methods were developed to assay plasma, whole blood and brain tissue. Since U-80,278A is pharmacologically potent and rapidly metabolised, high sensitivity was necessary. This requirement was complicated by the large number of potentially interfering compounds in the biological matrices and the small size of serial blood samples that could be taken from the rat.

Though the structure of some aminotetralins make them amenable to assay by a number of methods, including GC-MS and GC-NPD, HPLC with fluorescent or UV detection following solid phase extraction was the method chosen, and gave acceptable sensitivity with good sample throughput. Furthermore, techniques developed for U-80,278A could be adapted quickly for a range of substituted aminotetralins with different functional groups.

The analysis of U-80,278A, with a methyl analogue as internal standard is described in detail. Modifications of this method for other 2-aminotetralin analogues, and strategies for increasing sensitivity of assays, are discussed.

EXPERIMENTAL

Chemicals and Reagents.

U-80,278A and analogues were obtained from the Upjohn Company, Kalamazoo, Michigan, USA. Acetonitrile (CH_3CN), methanol (MeOH), water, trifluoroacetic acid (TFA), all HPLC grade, were obtained from Fisons, Loughborough, Leics., UK. Sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), Analar grade, were obtained from BDH, Poole, Dorset, UK. N, N-dimethyl-n-octylamine (DMOA) was obtained from Lancaster Synthesis, Morecombe, Lancs., UK. Control rat plasma, blood and brain tissue were prepared in-house.

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Equipment.

A range of Analytichem Bond Elut cartridges (100 mg size) and Vac-Elut ten place manifolds were obtained from Jones Chromatography, Hengoed, Mid Glammorgan, UK. A Denley bench top centrifuge, model 402, was obtained from Denley, Billingshurst, Sussex, UK. Eppendorf micro test tubes (1.5 and 0.5 ml sizes) were supplied by BDH. Sterilin specimen containers were obtained from Fisons.

Standard Solutions.

U-80,278A and other aminotetralin analogues were dissolved in either H_2O (free bases) or weak acid (salts) to give solutions of approximately 100 µg/ml. These solutions were further diluted with H_2O to provide solutions for spiking aliquots of control biological samples. All standard solutions were stored at 4°C.

Buffer Solution.

Phosphate buffer solution was made by mixing aqueous 0.05M Na_2PO_4 with aqueous 0.05M Na_2HPO_4 to pH 7.1. Solutions were stored at room temperature and prepared weekly.

Treatment of Biological Samples.

Plasma: blood samples (50 - 100 µl) from the rat tail or inferior vena cava were collected into 0.5 ml micro vials containing potassium EDTA. After centrifugation, the plasma was withdrawn into 0.5 ml test tubes. Plasma was stored at -20 °C prior to assay. Aliquots (25 - 50 µl) were spiked with 25 µl of internal standard solution(100 ng/ml) and diluted to 1 ml with H_2O prior to extraction.

Whole blood: blood from the inferior vena cava was collected into tubes containing K_2 EDTA and stored at - 20 °C prior to analysis. Aliquots (50 - 100 µl) were transferred to 1.5 ml test tubes, spiked with internal standard (as for plasma) and diluted to 1 ml with H_2O. After thorough mixing, samples were rapidly frozen by

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immersing the sample vial in liquid nitrogen and then thawing in warm water. The cycle was repeated at least five times to ensure complete haemolysis of the red blood cells. Haemolysates were centrifuged at 13,000 g, and the supernatants extracted immediately.

Brain: whole brains were weighed, placed into specimen containers and stored at -20 °C prior to analysis. Whole brains were placed into a weighed homogeniser. The specimen containers were rinsed with a small volume of phosphate buffer solution which was then added to the homogeniser vessel. The total weight of brain and buffer was made up to 3 g with phosphate buffer solution and homogenised over a 30 minute period. A weighed aliquot (50 - 100 mg) was removed to a mini vial, spiked with internal standard solution (as for plasma) and diluted to 1 ml with H_2O. After thorough mixing and centrifugation, the supernatant was extracted immediately.

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Preparation of Calibration Samples.

Plasma calibration samples were prepared daily over the range 5 - 1000 ng/ml U-80,278A by spiking 0.25 ml aliquots of control rat plasma with small volumes (up to 25 µl) of standard solution. After thorough mixing, three 25 µl or 50 µl aliquots were removed, the internal standard solution added, diluted to 1 ml with H_2O and extracted immediately. Whole blood was assayed against calibration curves prepared in control plasma.

Brain tissue was analysed against a calibration curve constructed by spiking control brain homogenate (50 - 100 mg) with standard solution over the range

10 - 250 ng U-80,278A per gram of brain. After thorough mixing weighed aliquots were removed, diluted to 1 ml with H_2O and extracted as for plasma.

Quality Control Samples.

Quality control samples to monitor the performance of the routine assay were prepared independently of calibration samples. Blank plasma was spiked with a small volume (up to a maximum of 0.5% of the total volume) of standard solution, thoroughly mixed and 50 µl aliquots transferred to 1.5 ml test tubes and stored at -20 °C. Five test tubes were removed for each analytical run, thawed, prepared and extracted as for the calibration and study samples.

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Extraction.

The required number of Vac-Elut boxes were loaded with Bond Elut diol cartridges. Cartridges were primed successively with 2 ml of CH_3CN, 2 ml of 60% CH_3CN, 0.1% TFA aqueous solution (the eluting solution) and 2 ml of H_2O.

Immediately after priming, prepared biological samples were drawn slowly though the cartridge using minimum vacuum. The cartridges were then rinsed with 2 ml of H_2O followed by 2 ml of aqueous 50% MeOH. The vacuum was increased to 20 - 25 mm Hg for 10 - 15 seconds to partially dry the cartridge.

U-80,278A and the internal standard were eluted with 0.6 ml of 60% CH_3CN 0.1% TFA, which was allowed to

percolate through the cartridge for 3 minutes before application of vacuum. After elution, the pressure in the Vac-Elut manifold was further reduced to evaporate eluates *in situ* to 150 - 200 µl.

HPLC.

The HPLC system consisted of a Waters 600 Multisolvent Delivery System pump, Waters 710B autosampler with low volume injection vials (Millipore Ltd., Watford, UK), an ICI TC1900 column oven (Anachem Ltd., Luton, Beds., UK), and a Perkin Elmer LS-4 fluorescence detector (Perkin Elmer Ltd., Beaconsfield, Bucks., UK). Data were captured on a Spectra Physics SP 4270 integrator/recorder (Spectra Physics Ltd., Hemel Hempstead, UK.).

The HPLC column was a Dupont Zorbax RX-C8, 250 X 4.6 mm id (Hichrom Ltd., Reading, UK.) maintained at 40 °C. The mobile phase was 20% CH_3CN, 0.05%TFA in H_2O, degassed with helium before use. The flow rate was 1.5 ml/min. The fluorescence detector was set at an excitation wavelength of 230 nm and an emission wavelength of 298 nm. 25 - 150 µl of extracts were injected on column and a typical analysis time was 16 - 20 mins.

The SP 4270 recorder/ integrator was configured to record peak height ratios of U-80,278A : IS (internal standard). Data were downloaded to a mainframe computer via a Spectra Physics ChromStation AT (Spectra Physics Ltd.). Further statistical analysis was carried out using the computer program SAS (SAS Institute Inc., North Carolina, USA). Linear regression was carried out

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using peak height ratio versus concentration using a weighted function, 1/concentration squared ($1/x^2$).

RESULTS

HPLC.

The aminotetralin analogues investigated are weak bases, (pka >8) which traditionally exhibit poor chromatography on C18 or C8 reversed phases without the addition of amine modifiers in the mobile phase. Zorbax TMS gave good chromatography for both U-80,278A and the internal standard with a mobile phase consisting of 20% CH_3CN, 0.1% dimethyl octylamine, 0.1% TFA. However, the addition of DMOA, whilst improving efficiency and peak shape, did not enhance selectivity with biological extracts, and comprimised electrochemical detection. UV detection at low wavelengths (<230 nm) necessary for analysis of other analogues and the use of thermospray detection could also be compromised by this mobile phase.

Zorbax phenyl provided good chromatographic characteristics without amine modifiers (fig. 2), but the high TFA concentration (0.4%) would limit its use to those analogues with strong UV maxima above 220 nm.

Zorbax CN stationary phase gave a lower though still usable efficiency with a similar mobile phase, (Table 1) but poor selectivity for biological extracts.

A number of "base deactivated" stationary phases was investigated (Table 2). The Zorbax RX-C8 stationary phase gave good efficiency and peak shape without the

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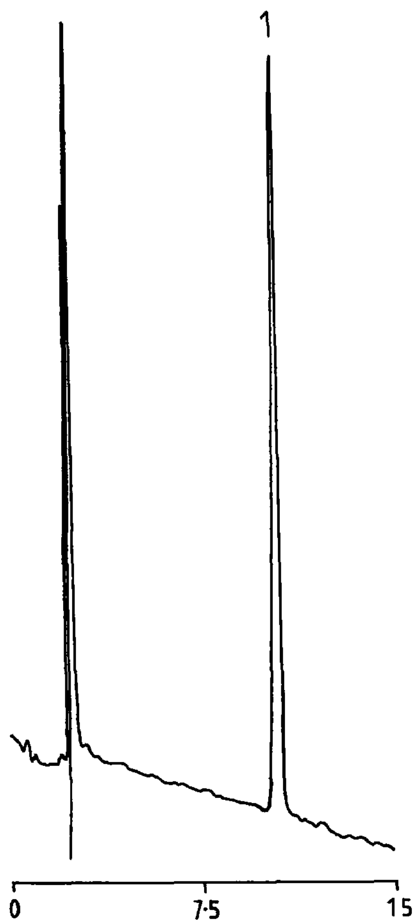

Figure 2

HPLC of U-80,278A (1) standard solution using Zorbax Phenyl stationary phase. Mobile phase 30% CH_3CN, 0.4% TFA in H_2O. Flow rate 1.5 ml/min, column temperature 40 °C and detection at 230 nm UV. Time scale is in minutes.

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Table 1

Summary of typical chromatographic parameters achieved during initial column screen. Each entry represents the optimum solvent composition of those tried.

Stationary phase	Mobile phase	K'	Efficiency (plates/ column)	Skew
Zorbax C8	35% CH_3CN 0.2% TFA	3.0	poor	-
Zorbax RX-C8	20% CH_3CN 0.05% TFA	3.1	13,700	1.17
Zorbax TMS	30% CH_3CN 0.1% TFA 0.1% Diethylamime	2.8	13,700	1.3
Zorbax CN	30% CH_3CN 0.2% TFA	2.2	9,600	1.08
Zorbax Phenyl	30% CH_3CN 0.4% TFA	3.8	14,000	1.25

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use of DMOA or high concentrations of TFA (Table 1, fig. 3). The selectivity (analyte peaks free of interferences) was also excellent for all biological extracts (figs. 4-7). HPLC with RX-C8 columns was used to support a number of animal pharmacokinetic studies where serial samples of 50 µl plasma were analysed. A lower limit of quantification of 5 ng/ml (CV 5.2%) was obtained (equivalent to 0.15 - 0.2 ng on column.

To improve the sensitivity of the assay further different column geometries were tried. Columns of 2.1 mm id (250mm in length) were used without modification to the autosampler or detectors, and gave acceptable efficiency and selectivity with biological extracts

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Figure 3

Standard solutions of U-80,278A (1) and the internal standard (2). Column Zorbax RX-C8, detection by fluorescence, excitation 230 nm and emission 298 nm. Mobile phase 20% CH_3CN, 0.05% TFA, flow rate 1.5 ml/min and column temperature 40 °C.

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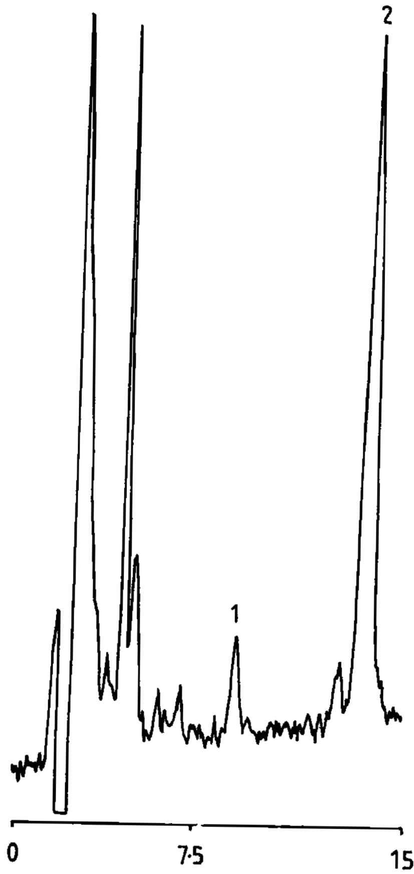

Figure 4

Extract of rat brain tissue 12 hours after dosing, containing 0.747 μg U-80,278A per gram of tissue. HPLC conditions as figure 3.

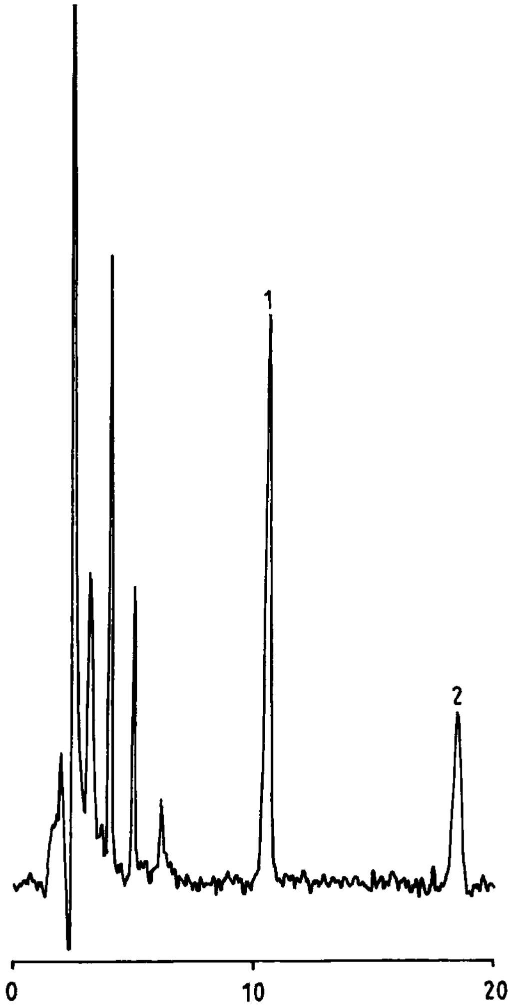

Figure 5

Extract of rat whole blood taken 12 hours after dosing, containing 63 ng/ml U-80,278A. HPLC conditions as figure 3.

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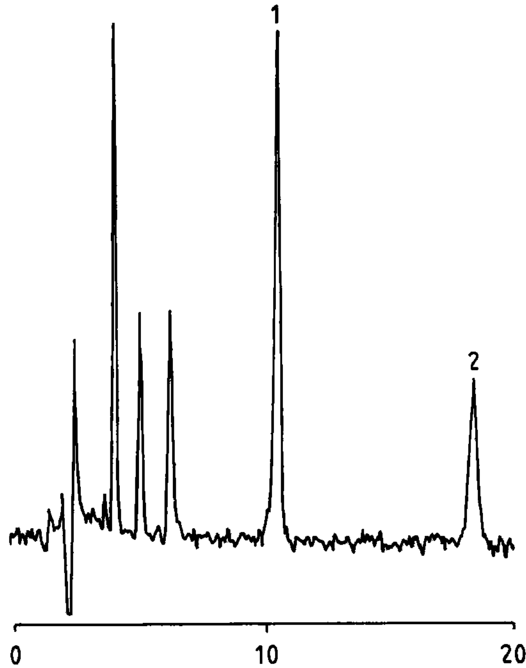

Figure 6

Extract of rat plasma taken 12 hours after dosing, containing 67 ng/ml U-80,278A. HPLC conditions as figure 3.

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(fig. 8), and a limit of quantification of 2 ng/ml (CV < 20%) was achieved in practice (c.a. 100 pg on column).

A sensitivity of 1.2 ng/ml (50 pg on column) was obtained using 1 mm id columns packed with phenyl stationary phase. These columns could not be used routinely. It was difficult to obtain reproducible performance from successive columns, and the introduction of biological extracts led to blocked

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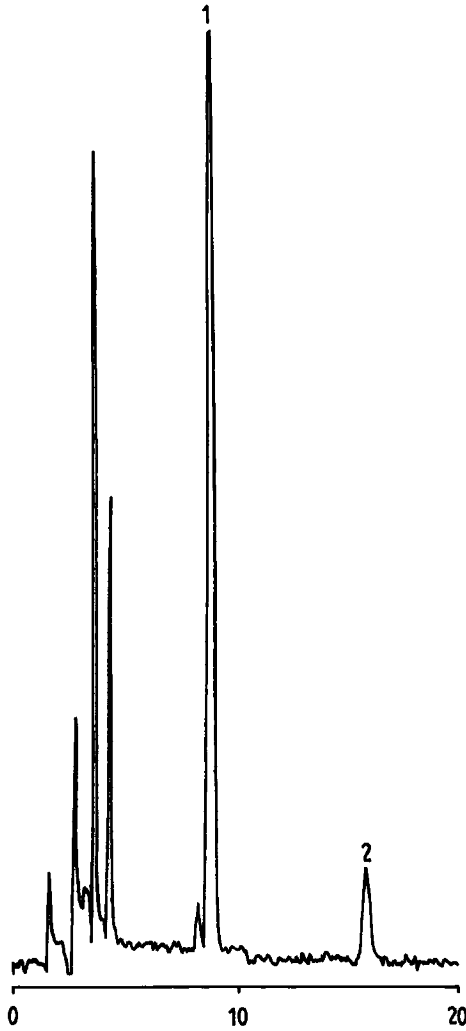

Figure 7

Extract of the contents of the rat gut, flushed from the animal 12 hours after dosing. The total drug remaining was 22.9 µg. HPLC conditions as figure 3.

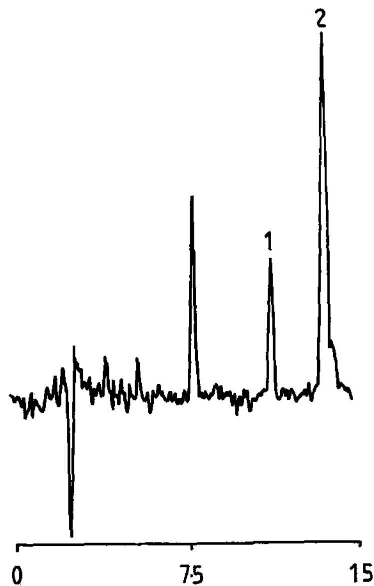

Figure 8

Extract of rat plasma 5 ng/ml U-80,278A calibration standard using a narrow bore HPLC column, Zorbax Phenyl 250 x 2.1 mm. HPLC conditions as figure three except flow rate used was 0.3 ml/min.

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frits, increasing back pressures and ultimately pump shutdown.

Detection.

The UV spectrum of U-80,278A had maxima at 220 and 270 nm but fluorescence detection (excitation 230 nm, emission 298 nm) provided greater sensitivity and selectivity in practice. Fluorescence detection was also effective for close analogues of U-80,278A. However, when other substituents (eg electron withdrawing) were

attached to the aromatic ring, this fluorescence was often lost. It was possible in a number of cases to excite the molecule at low wavelengths (200 - 210 nm) and achieve a fluorescence response. The sensitivity was not much greater than for low wavelength UV detection but selectivity was significantly better (fig. 9).

Extraction.

The extraction efficiency of U-80,278A and internal standard from plasma whole blood and brain homogenates was at least 89%. The procedure proved rapid, robust and the diol cartridge provided a useful contrast between the extraction phase and the HPLC stationary phase. This "mode" difference contributed to the interference free chromatograms seen with biological extracts (figs.4-7).

Reversed phase extraction cartridges, for example C18, C8, PH(phenyl) and CH (cyclohexyl) were effective for the extraction of U-80,278A and other aminotetralin analogues, but C2 (ethyl) and in particular the more polar phases, such as diol, and CBA used in ion exchange mode, provided better selectivity for all biological matrices.

Minor modifications to the extraction procedure were required in some cases to retain the selectivity and efficiency of the rinsing and elution steps. Volumes of solvents were unchanged, but variation of the proportion of methanol, or acetonitrile, or higher TFA concentration (upto 0.5%) were effective in all cases.

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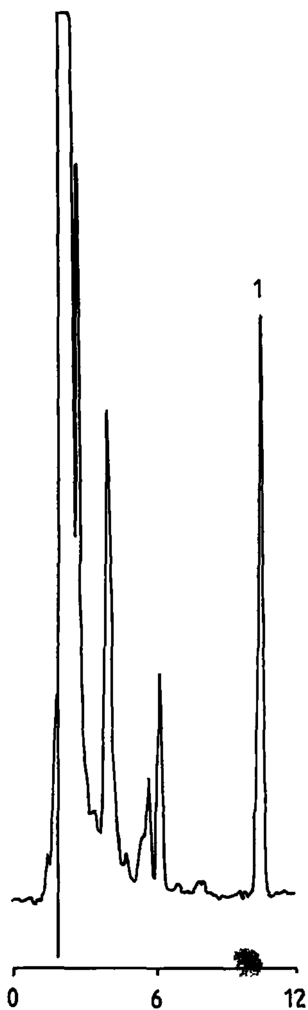

Figure 9

Example chromatogram showing a whole blood extract chromatographed using a Zorbax RX-C8 column and using fluorescence detection at 205 nm excitation and 300 nm emission. Other chromatographic conditions as figure 3. Component of interest was a close analogue of U-80,278A.

Stability of Extracts.

U-80,278A was stable in fresh rat plasma for at least three hours at room temperature. Extracts could be stored at -20 °C for seven days without loss of analyte or the appearance of interfering peaks. Spiked biofluids could be subjected to repeated freeze thaw cycles without loss of analyte.

Linearity and Repeatability.

The assay for U-80,278A was linear over the range 2 to 1000 ng/ml in plasma or whole blood and from 13 to 259 ng/gram in brain tissue. The coefficient of regression (r) was better than 0.99 (n>15) in all cases. The coefficient of variation (CV) within assay was generally better than 10% at all levels. Inter assay CV was better than 15% at all levels (4.6 mm id columns).

Repeatability of the assay during routine application using "conventional" and narrow bore HPLC methods was acceptable.

DISCUSSION

The assay for U-80,278A and the internal standard described has been used successfully to support many small animal studies, and the experience gained in routine application, and during development, where many options of extraction cartridge and HPLC stationary phase were explored, has resulted in an assay that can quickly be adapted for differently substituted aminotetralins.

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Table 2

Comparison of "base deactivated" columns for the chromatography of U-80,278A. Each column was assessed with acetonitrile, tetrahydrofuran and methanol as the organic modifier.

Stationary phase	Mobile phase	K'	Efficiency (plates/ column)	Skew
Zorbax RX	27% CH_3CN 0.1% TFA	2.5	11,080	1.08
Kromasil C8	24% CH_3CN 0.1% TFA	3.3	10,130	1.14
Hypersil BDS-C8	24% CH_3CN 0.1% TFA	2.8	7,677	1.43
LiChrosorb RP-Select B	22% CH_3CN 0.1% TFA	5.0	10,244	1.42
Deltabond C8	33% MeOH 0.3% TFA	2.2	7,270	1.68

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For some U-80,278A analogues, detection was only possible by UV at wavelengths of 230 nm or less. In these circumstances, the selectivity of extraction and chromatography was all important. Minor modifications to the organic modifier strength or the concentration of TFA used in the mobile phase were sufficient to achieve efficient chromatography for all the analogues with the Zorbax RX-C8 column. To obtain acceptable selectivity with other stationary phases, it was sometimes necessary to change the organic modifier (Table 2).

Electrochemical detection was investigated but no real improvement in sensitivity or selectivity was seen. Thermospray mass spectrometry was used to good effect

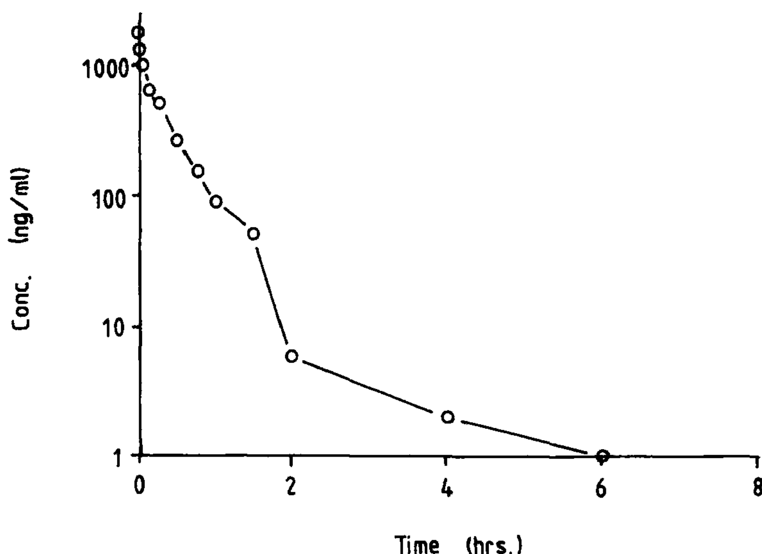

Figure 10

Example of rat plasma profile, log concentration U-80,278 verus time. Animal dosed iv (inferior vena cava) at 5 mg/kg free base.

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for analyte identification without modification to the chromatography.

During the development of the described assay, a large number of aminotetralin analogues was investigated, and a "data base" of their chromatographic and other characteristics compiled. As assays for each new analogue were developed, the data base allowed effective choice of internal standard to be made, and expedited optimisation of extraction and HPLC conditions of a new assay. In this way, assay development kept up with the needs of the animal experiments, and importantly, an abbreviated method validation procedure

could be used without compromising established GLP guidelines.

CONCLUSION

The assay has been successfully used to support the research programme for U-80,278A. The sensitivity and selectivity have allowed accurate determination of important pharmacokinetic parameters (fig. 10). With modification to extraction and HPLC conditions, the assay can be used for other substituted aminotetralins.

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