HPLC Quantitation of a Very Hydrophilic 3-Hydroxypyridin-4-one Chelator Using a Simple Separation Procedure and the Baseline File Subtraction Method

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Abstract

The lack of a method to isolate very hydrophilic 3-hydroxypyridin-4-ones (HPs) from blood has prevented determination of their pharmacokinetics. The objective of this study is to develop a method to quantitate these compounds. A simple sample preparation method coupled with high-performance liquid chromatography is used to quantitate 1-[ethan-1-ol]-2-methyl-3-hydroxypyridin-4-one, a very hydrophilic HP, in plasma. Plasma proteins are precipitated by trichloroacetic acid. The baseline file subtraction method is used to improve the resolution of this HP in the presence of interfering chromatographic peaks that could not be resolved from the HP by the methods investigated. The method is used to determine the pharmacokinetics of this HP in rabbits. The precision of the pharmacokinetic results is comparable or better than the results obtained from seven more lipophilic HPs that were separated by a published method. The new method is slightly modified and used in a study of the pharmacokinetics of this HP in the rat, and precision is comparable with results obtained with two more lipophilic HPs determined by the published method. Baseline file subtraction is useful when other methods cannot be used to adequately resolve a hydrophilic analyte from coeluting interfering substances.

Introduction

The 3-hydroxypyridin-4-ones (HPs) are effective iron and aluminum chelators in vitro and after oral administration (1–5). They are potential alternatives to the parenterally effective chelator desferrioxamine. Their structural simplicity (Figure 1) and the capability to introduce a wide variety of substituent groups at sites not involved in chelation allow the inexpensive production of a large number of HP analogues with a wide range of lipophilicities. A positive correlation between lipophilicity and toxicity was reported (6). However, the relationship between lipophilicity and the pharmacokinetics of the HPs has not been described. 1,2-Dimethyl-3-hydroxypyridin-4-one (CP20) (also known as L1, HP4A, Hdpp, DMPH, CGP 37 391, deferiprone, CAS Registry #30652-11-0), which has an octanol/aqueous distribution coefficient ($D_{o/a}$) approximately equal to 0.09 (1), demonstrates good chelation activity (2–5). These results encourage the study of more hydrophilic HPs because of their predicted lower toxicity (6). However, good oral absorption may be prevented by their hydrophilicity, necessitating the determination of their oral bioavailability. The inability to separate very hydrophilic HPs from chromatographically similar blood components has prevented the study of their oral bioavailability and pharmacokinetics.

This report describes attempts to separate a very hydrophilic HP, 1-[ethan-1-ol]-2-methyl-3-hydroxypyridin-4-one (CP40) (EL1NEt2'(OH)), from blood components that interfered with its chromatographic resolution. The method developed utilizes a simple, rapid, inexpensive sample preparation method prior to high-performance liquid chromatographic (HPLC) analysis. Because the conditions investigated did not resolve CP40 from coeluting blood components, it was quantitated as the peak obtained when the chromatogram of a sample obtained from the same subject before administration of CP40 was subtracted from the chromatogram of the sample containing CP40, using the baseline file subtraction method.

Experimental

Chemicals

The HPs were prepared as hydrochloride salts in the laboratory of Robert C. Hider, Kings College, London, United Kingdom.

Apparatus

The HPLC system used for these studies was composed of an LC-600 liquid chromatographic pump, an SCL-6B system controller, an SIL-6B autoinjector with a 20-µL injection loop, and an SPD-10A ultraviolet spectrophotometric detector, all from Shimadzu Scientific Instruments (Columbia, MD). A Shandon Hypercarb-S graphitized carbon packed analytical

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column (Alltech; Deerfield, IL) and a pellicular C_{18} filled guard column (Alltech) were used. The system was controlled and data were analyzed by the EZChrom Chromatography Data System (Version 2.1) from Shimadzu and an IBM PS/2 Model 40 SX computer. The flow rate was 1.0 mL/min. The absorption wavelength was at 270 nm.

Procedures

The HPLC analysis of HPs was described (7) using a Shandon Hypercarb column and a mobile phase composed of an 86:14 (v/v) mixture of 10mM NaH_2PO_4 and acetonitrile (ACN), which contained 2mM EDTA and was adjusted to pH 3.0 with phosphoric acid. Prior to HPLC analysis, the HPs were extracted into methylene chloride from blood in a pH 7.0 buffer. With this method, the minimum quantitatable concentration of seven HPs that are more lipophilic than CP40 was 250 to 750 ng/mL (8). For these seven HPs, extraction efficiencies (8) ranged from 11.5% for CP20, the most hydrophilic HP, to 87% for 1,2-diethyl-3-hydroxypyridin-4-one (CP94) (El1NEt), which has a $D_{o/a}$ of 2.1 (1). However, CP40, which has a $D_{o/a} < 0.002$ (6), could not be extracted into methylene chloride. Therefore, the following procedures were investigated in an attempt to separate CP40 from blood components that interfered with its analysis.

Extraction of CP40 from blood into liquids that are more hydrophilic than methylene chloride was investigated. The liquids studied were *n*-butanol, ethyl acetate, chloroform, and a 10:90 (v/v) *n*-butanol and hexane mixture.

The capability of molecular sieving to remove the blood components that interfered with the analysis of CP40 was investigated. Plasma ultrafiltrate was obtained from blood spiked with CP40 using Amicon Centrifree devices (30,000 MWCO). Sample refiltration through Amicon Microcon devices (3000 MWCO) was also investigated.

Solid-phase extraction was extensively investigated. Strong and weak cation- and anion-exchangers, as well as several mixed-phase particle bed devices, were investigated. A total of approximately 50 different retention and elution conditions was evaluated.

The above methods did not adequately isolate CP40 from interfering substances in blood. Therefore, we evaluated a method that produces a protein-free plasma. The lack of plasma protein binding of CP40 (8) enabled the study of plasma rather than whole blood. A trichloroacetic acid (TCA) solution was added to the plasma sample at a final concentration of 2.5% to generate a protein-free supernatant. This supernatant was chromatographed using several mobile phases, but CP40 could not be adequately resolved from the interfering blood components.

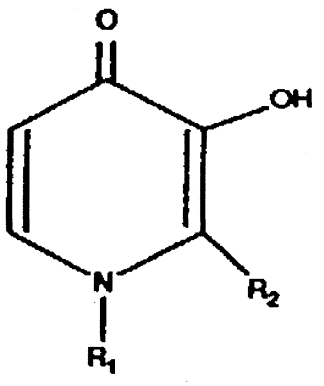

Figure 1. Chemical structure of the 3-hydroxypyridin-4-ones. Metal chelation occurs at the 3-hydroxy and 4-keto positions. For CP20, CP94, CP40, and CP102, R_1 and R_2 are Me and Me, Et and Et, EtOH and Me, and EtOH and Et, respectively.

Suggested methods to improve resolution (9) were investigated. The capacity factor (k') was optimized; mobile phase, stationary phase, and sample chemistry changes were investigated to improve separation; and changes to improve the number of plates (N) were investigated. Although this resulted in an improvement of resolution, baseline resolution was not achieved.

To further investigate methods to improve the resolution and purity of CP40 in the presence of coeluting substances, the absorbances of CP40 and the interfering peaks were determined as a function of absorption wavelength. This failed to reveal a wavelength at which absorption was significantly greater for CP40 than the interfering peaks.

Given the inability to adequately separate CP40 from interfering substances, the capability of the baseline file subtraction method to improve resolution was investigated. The approach used was to process and analyze a sample that did not contain CP40 (baseline sample), as well as an analytical sample that contained CP40. The baseline sample chromatogram was then subtracted from the analytical sample chromatogram. To prepare samples for method development, CP40 was added to blood 1 h before plasma was obtained. Protein-free plasma, produced by TCA addition and centrifugation, was chromatographed using a pH 3.0 mobile phase composed of an 88:12 (v/v) 10mM NaH_2PO_4–ACN mixture containing 0.66% triethylamine (TEA) and 4mM EDTA.

To validate this method, rabbit blood samples were spiked with CP40 at each of five concentrations, incubated, and centrifuged to obtain plasma, which was frozen. This blood sample was a mixture of blood samples obtained from five male rabbits that individually generated similar baseline files. At a later date, 10 replicate samples of each concentration were thawed, as well as aliquots of the pooled blood to which no CP40 had been added. Protein was precipitated by TCA addition, and the supernatant was chromatographed to determine intraday variability. To determine chromatographic stability, the baseline sample was processed first. The spiked samples were then processed. The file of the baseline was subtracted from the chromatogram of each of the spiked samples to determine intraday variability. These results were compared with those obtained when the baseline sample was analyzed before every seven spiked samples and the baseline sample file was subtracted from the chromatogram of each of the seven spiked samples. A measure of chromatographic noise was obtained from the average of the absorbances at the retention time of CP40 for five independently processed replicates of rabbit blood that did not contain CP40 after the baseline chromatogram was subtracted from each of these chromatograms. To determine the interday variability of this assay, quality control samples containing 75,000, 25,000, 5000, and 1250 ng CP40 per millilter were prepared in a mixture of blood obtained from several rabbits, frozen, and then processed with the samples of the pharmacokinetic study.

The above sample preparation procedure and chromatographic conditions were used to quantitate CP40 in 15–16 blood samples obtained at times ranging from 5 min to 24 h after dosing each of eight male rabbits with 0.45 mmol/kg of CP40 HCl by both the intravenous (iv) and per os (po) (i.e., oral) routes. Blood samples were collected, centrifuged to obtain plasma, frozen, subsequently thawed, rendered protein free by TCA precipitation, and analyzed by HPLC using the background file subtraction method. A blood sample was also obtained from each rabbit immediately before each dosing of CP40. This predosing (baseline) blood sample was prepared for HPLC with the post-dosing samples. It was processed by HPLC before the post-dosing samples to generate the baseline file. The concentration of CP40 in post-dosing samples was determined by comparison of sample peak height with a standard curve generated from similarly processed standards prepared in the pooled blood used for the intra- and interday variability studies.

The HPLC results obtained were used to calculate the pharmacokinetics, including systemic availability, of CP40. The concentration of CP40 was plotted versus time. The nonlinear regression program RSTRIP (10) was used to determine pharmacokinetic parameters by fitting an exponential equation to each experimental plasma CP40 concentration-versus-time profile. The area under the concentration-versus-time curve (AUC) was determined by the trapezoidal method using extrapolation to infinity. The AUC, area under the moment curve (AUMC), mean residence time (MRT), and elimination half-life were determined by RSTRIP. The volume of distribution at steady-state was calculated as $(\text{dose} \times \text{AUMC}_{iv})/(\text{AUC}_{iv} \times \text{AUC}_{iv})$. Systemic clearance was calculated as $\text{dose}/\text{AUC}_{iv}$. The mean absorption time was calculated as $\text{MRT}_{po} - \text{MRT}_{iv}$. Systemic availability was calculated as $\text{AUC}_{po}/\text{AUC}_{iv} \times 100\%$.

Results and Discussion

We developed and validated a separation and HPLC method to quantitate a very hydrophilic HP, CP40. The method enabled determination of its pharmacokinetics in rabbits and rats. The method consists of a simple sample preparation step and HPLC quantitation using the baseline file subtraction method. Because of the inability of the other procedures that were investigated to resolve CP40 from coeluting substances present in blood, the baseline file subtraction method was adopted.

Using liquid–liquid extraction, we attempted to extract CP40 into more hydrophilic liquids than methylene chloride, but this method failed to yield more than 10% recovery. Attempts to use liquid-phase extraction on an HP analogue that is more lipophilic than CP40, 1-(2-hydroxyethyl), 2-ethyl-3-hydroxypyridin-4-one (CP102; $D_{o/a} = 0.22$ (3)), including formation of a hydrophobic ion pair to transfer the HP into the organic phase where it would then be methylated and retained, were also unsuccessful (R. Hider, King's College, UK, personal communication, 1994). Therefore, the use of liquid–liquid extraction to prepare blood samples for HPLC analysis has not been found to be applicable to very hydrophilic HPs.

The plasma ultrafiltrate obtained from the Amicon Centrifree device had substances that coeluted with CP40. Sample refiltration through an Amicon Microcon device removed most of the interfering peaks but did not do so sufficiently for HPLC analysis using the described procedures (7). We were unable to find a mobile phase that would resolve CP40 from the interfering blood components in these ultrafiltrates. The baseline file subtraction method might have allowed the use of sample preparation by ultracentrifugation, but the TCA method to produce protein-free plasma, adopted below, requires approximately 25 to 35% as much time and avoids the cost of ultracentrifugation devices (greater than or equal to $2.50 per sample).

Solid-phase extraction devices containing strong and weak cationic groups most effectively retained CP40, suggesting that it was acting as an anion. However, its recovery in a volatile eluent was not more than 6% under any condition tested.

Sample preparation by TCA addition to plasma produced cleaner chromatograms than those obtained with ultrafiltrates. To determine the recovery of CP40 from the TCA procedure, CP40 was added to rabbit blood. TCA was added to prepare protein-free plasma that was analyzed by HPLC and compared with protein-free plasma to which CP40 had been directly added. The recovery was 93%.

However, interfering substances still coeluted with CP40 when a mobile phase composed of an 86:14 (v/v) mixture of 10mM NaH_2PO_4 and ACN with 2mM EDTA ($k' \approx 2$) was used. Therefore, numerous steps were taken to try to improve resolution. To adjust the k' into the optimal range, the mobile phase composition was changed to an 88:12 (v/v) NaH_2PO_4–ACN mixture, which produced a k' of 6. Resolution was 0.4. To improve selectivity, mobile phase chemistry changes were investigated. The retention times of CP40 and the interfering peaks using the aforementioned mobile phase, as well as a mobile phase containing methanol at a concentration that maintained the same k' (a 90:10 [v/v] mixture of 10mM NaH_2PO_4 and methanol), were compared. The results suggested that an 86.2:10.6:3.2 10mM NaH_2PO_4–ACN–methanol mobile phase should best resolve these peaks. However, no improvement was obtained with this mobile phase. The ion-pairing agent TEA was added (0.66%) to the latter mobile phase to interact with the anionic group of CP40. Resolution increased to 0.6. Changes in stationary phase chemistry were not tested because previous studies had shown the Hypercarb column to perform best among the several columns tested for resolution of the HPs (11). Increasing the mobile phase phosphate concentration to 200mM to decrease ionization of CP40 did not improve resolution. Doubling the mobile phase flow rate in an attempt to increase the plate number produced excessive pressure. It would have been necessary to increase the column length 4 times to double the plate number, which was not practical. The supplier of Hypercarb columns (Alltech) does not list a smaller particle size, ruling out this approach to improve the plate number.

As other methods failed to resolve CP40 from interfering substances, the baseline file subtraction method was investigated. This method subtracts a file containing a baseline chromatogram, the baseline file, from the sample chromatogram.

Although the baseline file subtraction method has been used in HPLC techniques involving temperature programming and gradient elution, there are no reports of its use to eliminate interference from coeluting substances with stable temperature isocratic HPLC. Figure 2, panel A, shows a representative baseline chromatogram obtained from rabbit blood. The three peaks are from substances in blood that eluted shortly before CP40, at the same time as CP40, and shortly after CP40, which is noted as CP40 in panel B. Panel B shows the chromatogram from a sample containing approximately 30,000 ng CP40 HCl/mL, which eluted with a substance that appears as a shoulder on the leading edge of the CP40 peak (peak 1) and with another substance that eluted shortly after CP40, peak 3. The resolution of CP40 was approximately 0.6 from the preceding peak and 0.9 from the following peak, respectively. The plate number was approximately 860. Subtraction of the baseline file (Figure 2, panel A) from the sample chromatogram (Figure 2, panel B) produced the baseline-subtracted file shown in Figure 2, panel C. Baseline file subtraction removed the shoulder preceding CP40; removed the peak coeluting with CP40, which greatly improves its purity; and improved the resolution from the peak following CP40 to approximately 1.4 (and improved the plate number to approximately 1085). These plate number values compare with approximately 3500 for the four components of the test solution of the Hypercarb column when new, according to Shandon, and approximately 1300 for the test solution components after completion of this study.

Analysis of 10 replicates of 75,000, 25,000, 5000, 2500, and 1250 ng CP40 HCl/mL (365, 120, 24, 12, and 6μM) yielded intraday relative standard deviations (RSDs) of 1.5, 2, 9, 10, and 20%, respectively, when the baseline subtraction method was not used. The RSDs for these concentrations of CP40 were 1.6, 2, 6, 5, and 8%, respectively, when the baseline file subtraction method was used.

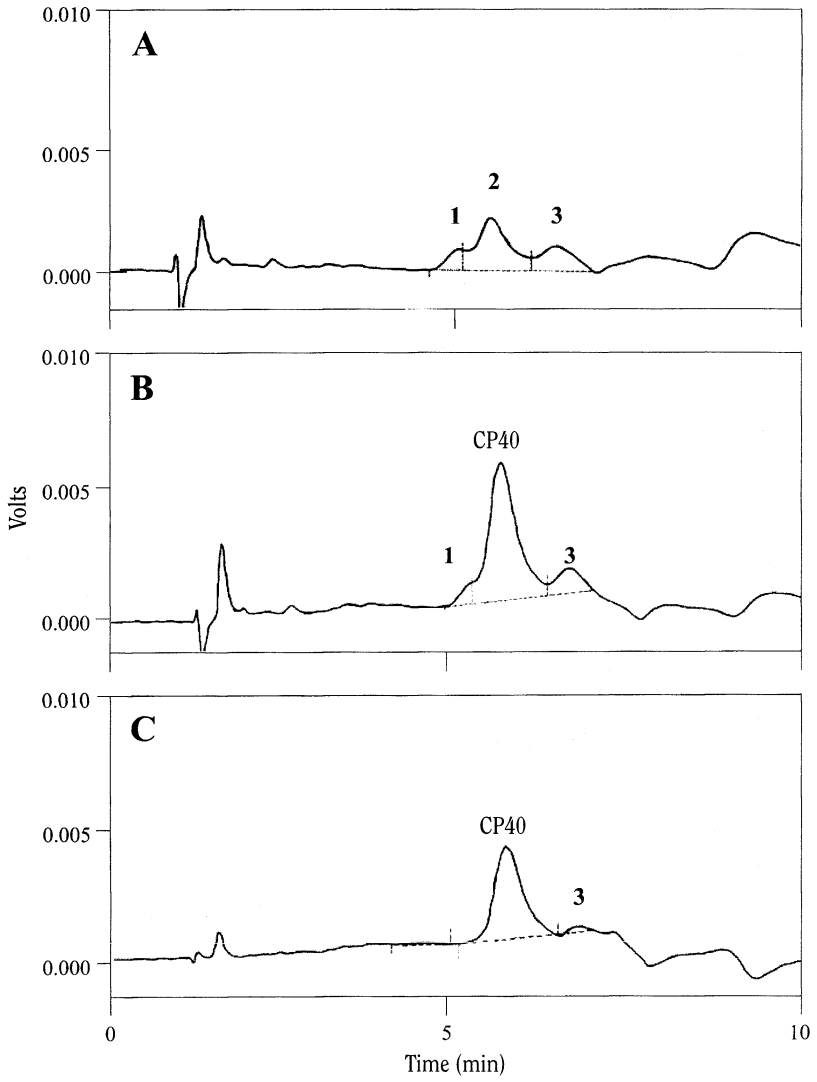

Figure 2. Panel A: Chromatogram of a protein-free plasma sample obtained from rabbit blood that did not contain CP40 (baseline sample). Panel B: Chromatogram of a protein-free plasma sample obtained from the blood of the same rabbit as in Panel A, 3 h after administration of 0.45 mmol/kg of CP40 HCl intravenously, which was determined to have approximately 30,000 ng CP40/mL. Panel C: The baseline-subtracted file that resulted from the subtraction of the chromatogram in panel A from the chromatogram in panel B. Peaks 1, 2, and 3 are from substances that eluted shortly before CP40, at the same time as CP40, and shortly after CP40, respectively.

Subtraction of baseline chromatograms obtained before every seven intraday variability samples did not yield a better RSD for the samples than subtraction of the one baseline chromatogram processed before all of the samples. Therefore, the latter method was used in the pharmacokinetic study. The lack of improved precision after subtraction of baseline files, which were obtained in close temporal proximity to the samples, compared with subtraction of the baseline file obtained before all samples in a batch shows the stability of the HPLC conditions. The measure of chromatographic noise, which was obtained by subtraction of the baseline file from the chromatographic files of independently processed replicates of the baseline sample, showed that a sample with 3 times the noise would have 900 ng CP40 HCl/mL.

The pharmacokinetics of CP40 in the rabbit were determined using the TCA precipitation procedure and HPLC with the baseline file subtraction method. Only blood samples having greater than or equal to 5000 ng CP40 HCl/mL were included in the pharmacokinetic analyses. This value was based on the intraday variability (less than or equal to 8% for 1250 to 75,000 ng/mL), interday variability (less than or equal to 12% for 5000 to 75,000 ng/mL), and chromatographic noise (900 ng/mL). The introduction of 20 μL of sample containing 5000 ng CP40 HCl/mL onto the HPLC column would contain 93 ng of CP40 HCl (5000 ng/mL × 0.02 mL × 0.93 efficiency). The sample preparation method used to quantitate seven more lipophilic

analogues of CP40 in blood, enabled analysis of 250 to 750 ng HP per milliliter blood (18 to 44 ng injected onto the HPLC column). This method involved liquid-phase extraction of the HP from 1 mL blood in a pH 7.0 buffer into methylene chloride and reconstitution into 100 µL (8). Therefore, the present HPLC method, applied to the most hydrophilic HP studied, was only a few fold less sensitive than the liquid-phase extraction method for more lipophilic HPs. However, the potential to concentrate the HP 10 times during extraction into methylene chloride was lost with the TCA procedure. The method we developed enabled quantitation of CP40 in rabbit blood for 6 to 8 h after dosing. This was comparable with or greater than the period of time that the seven more lipophilic HPs could be quantitated in blood after the same dose, using a published method (7).

The RSD for the pharmacokinetic parameters obtained with CP40 and summaries of RSDs obtained for the seven more lipophilic HPs are shown in Table I. The results show that the precision of the pharmacokinetic parameters estimated for CP40 was comparable with the precision of the results obtained for seven more lipophilic HPs (8), suggesting that this method does not introduce systematic errors that are not present in the method used for the other HPs.

The systemic availability of CP40 was 73%. This was as good as or better than the other seven HPs studied, which ranged from 30 to 74%. There was no correlation between lipophilicity and the systemic availability of this family of chelators. The TCA sample preparation procedure and HPLC using the baseline file subtraction method enabled the addition of CP40 to a study examining the relationship between lipophilicity and systemic availability after oral administration. The addition of CP40 extended the range of lipophilicity of HPs that could be investigated by greater than or equal to 50-fold and showed that very hydrophilic HPs have the capability to be absorbed orally. Very hydrophilic HPs warrant further study as chelators of the trivalent hard metals (iron and aluminum) because they are predicted to be less toxic than more lipophilic HPs (6).

These results were obtained from male rabbits. Chromatograms obtained from female rabbit blood processed and chromatographed under the same conditions were quite different. These results suggest that the baseline and analytical samples must come from subjects of the same gender, if not from the same subject, when the analyte is being studied in blood and the baseline file subtraction method is used.

Table I. %RSD for Pharmacokinetic Parameters Obtained With CP40 and for Seven More Lipophilic HPs*

Parameter	RSD of CP40	RSD of lipophilic HPs*	
		Range	Mean
Area under curve*	38	22–56	32
Volume of distribution*†	30	23–65	38
Mean residence time*	27	23–77	47
Systematic clearance*	25	14–46	30
Half life*	32	33–124	59
Mean absorption time	83	33–140	90
Systematic availability	26	24–57	42

* Intravenous.
† At steady state.

We attempted to use the method to separate and quantitate CP40 in rat blood. The mobile phase used to analyze CP40 in protein-free rabbit plasma did not adequately resolve it from interfering substances. A resolution of 0.6 was obtained with protein-free rat plasma using the following conditions: the TEA content was increased to 1.2%, the mobile phase composition was changed to a 91:9 (v/v) mixture of 10mM NaH_2PO_4–ACN, and a flow rate of 1.25 mL/min was used. Substances coeluted with CP40, producing a small increase above baseline. Baseline file subtraction reduced the interference from the interfering substances, resulting in a resolution of 1.3 and baseline resolution. Intraday variability of 75,000, 12,500, 2500, and 500 ng CP40 HCl/mL (10 replicates per concentration) resulted in RSDs of 11, 6, and 39% before baseline subtraction for the three highest concentrations. Resolution could not be obtained for 500 ng CP40 HCl/mL. After baseline file subtraction, RSDs were 10, 5, 21, and 36%, respectively. These conditions were used to determine the pharmacokinetics of intravenously administered CP40 in rats (12). The RSDs of the pharmacokinetic parameters obtained with CP40 using this method were comparable with those obtained with CP20 and CP94 using the described method (7).

The baseline file subtraction method was found to improve chromatographic results when other methods could not adequately resolve CP40 from coeluting substances. Application of this method to blood samples may necessitate obtaining baseline samples from subjects of the same gender, or optimally, from the same subject on the same day as the samples to be studied. Slight mobile phase modifications would be necessary to maximize the resolution of the analyte peak from interfering peaks for any application other than a replication of our study, based on the differences we observed in the chromatograms of blood samples from male and female rabbits and the mobile phase modifications we found necessary to maximize peak resolution in samples from rats.

Conclusion

This is the first report describing the baseline file subtraction method applied to stable temperature isocratic HPLC. However, there are others who are using the method to conduct animal studies of drugs that could not be resolved from interfering peaks (John Brady, Shimadzu, personal communication, 1995). Although the baseline file subtraction method lacks the precision of a separation technique that produces baseline resolution, its use may enable analyses that are not otherwise possible with HPLC.

Acknowledgments

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