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Direct analysis of the dopamine agonist (−)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride in plasma by high-performance liquid chromatography using two-dimensional column switching

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ABSTRACT

A reversed-phase, two-dimensional, liquid chromatographic method incorporating column switching and electrochemical detection was used for the direct analysis of the dopamine (D2) agonist (−)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride in plasma. Sample work-up consisted of addition of internal standard, filtration, then direct injection of the plasma sample onto an internal surface reversed-phase (ISRP) guard column where the dopamine agonist and internal standard were separated from plasma proteins. An automated pneumatic valve was then used to switch to a stronger eluent which stripped the retained substances from the ISRP support onto a C_{18} analytical column where the analytes were separated from endogenous biological interferences. A dual-electrode electrochemical detector was used to minimize interferences and provide the desired sensitivity. The method has a detection limit of 1.5 ng/ml and requires a total assay time of 20 min per plasma sample. The method is linear from 1.5 to 1000 ng/ml and yielded >80% drug recovery for plasma concentrations >10 ng/ml. Precision for the method at 100 ng/ml yielded a relative standard deviation of 4.4%. Reproducibility was within 6.5% on a 20 ng/ml spiked plasma sample assayed on different days by different people. The method has succesfully been applied to human plasma samples and for pharmacokinetic studies in rats and monkeys.

INTRODUCTION

Analysis of drugs in plasma using high-performance liquid chromatography (HPLC) typically involves the use of solid or liquid-phase extraction procedures to remove plasma proteins which can clog HPLC columns and deteriorate chromatographic performance. With large numbers of samples, these procedures rapidly become tedious and undesirable due to the extensive sample work-up and manpower required.

Recently, in an effort to simplify and reduce the time required to assay drugs in plasma, direct injection techniques have been explored. These include automated

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liquid–liquid or liquid–solid extraction [1–5], microinjection [6], micellar chromatography [7,8], and column-switching [9–16] methods. Of these, column switching has received the most attention in the literature. A majority of these column-switching methods incorporate two reversed-phase C_8 or C_{18} columns, one to retain analytes and exclude plasma proteins, the other for analyte separation and quantitation [9–11,13,15]. Several of these methods report nanogram level detection limits for drugs in plasma through the use of electrochemical detection [10,11,13,15]. While these methods can be successfully used to directly analyze drugs in plasma, problems such as protein precipitation [17] and column clogging can occur during routine use [18,19]. Also, routine injections of untreated plasma onto alkyl bonded phase porous silica will significantly damage microparticulate columns [19–21]. In an attempt to circumvent these problems associated with direct plasma injection, multi-mode or internal surface reversed-phase (ISRP) stationary phases have recently been designed [22–25]. These supports have novel properties, retaining small molecules such as drugs but allowing macromolecules such as plasma proteins to be sterically excluded. The ISRP support can thus serve to accomplish a sample clean-up function as well as provide an analytical separation of drugs in plasma.

ISRP supports which strongly retain hydrophobic drugs [19,23] require a weak initial mobile phase to remove plasma proteins without causing their precipitation, then a second mobile phase with higher concentration of organic modifier to elute the retained drug. However, mobile phase gradients are undesirable for the analysis of large numbers of samples because of the need to re-equilibrate the ISRP column prior to each injection. To eliminate the need for mobile phase gradients and also to use ISRP supports as sample concentration devices, column-switchting ISRP methods have been developed [26–28]. These methods have the advantage that the chromatographer can choose from a wide variety of stationary phases to accomplish the analytical separation. This two-dimensional methodology increases the flexibility of the ISRP approach to drug analysis substantially. The published methods involving ISRP columns and column switching [26–28] incorporate UV detection techniques, however, and there have been no reports of the use of ISRP column switching combined with electrochemical detection, which is the subject of this report.

It was of interest to us to develop a sensitive and rapid assay for (−)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride (I) in plasma because this drug exhibits potent dopamine (D2) agonist properties, and has potential applications in the treatment of Parkinson's disease [29–31]. The method requires a low limit of detection because of the drug's high potency and expected first-pass metabolic effect [31]. Recently, two electrochemical HPLC methods have been reported [33,34] for the determination of I in plasma. Both methods, however, require time-consuming, multi-step solid-phase extraction procedures which can easily introduce contaminants into samples. To overcome this hindrance, we developed an alternative analysis method involving an ISRP and C_{18} stationary phases using column switching and electrochemical detection.

The ISRP support used in this work has a hydrophobic tripeptide internal surface (glycyl-1-phenylalanyl-1-phenylalanine) and hydrophilic diolglycine external surface [23] which preferentially retains non-polar analytes. The synthesis, physico-chemical properties, and some applications of this commercially available 'Pinkerton' column have recently been published [19,23,37–40]. This particular ISRP support was selected because hydrophobic drugs such as I were strongly retained, allowing effective removal of proteins from the plasma. This paper describes a unique HPLC assay for I which may also be used for other, similar, hydroxytetralin compounds. The method requires very small volumes of plasma and can rapidly analyze large numbers of samples.

EXPERIMENTAL

Materials

(−)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride (I) was synthesized by Ethyl Chemical (Baton Rouge, LA, U.S.A.) and was determined to be 99.1% pure by differential scanning calorimetry. The hydroxytetralin internal standards, II and III, were synthesized by Whitby Research (Irvine, CA, U.S.A.) and were >99% pure by HPLC. High-purity grade Burdick & Jackson acetonitrile was purchased from Baxter Healthcare (Muskegon, MI, U.S.A.) Water was deionized using a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). Monobasic and dibasic sodium phosphate were Fisher Scientific reagent grade (Fairlawn, NJ, U.S.A.). Rainin P-1000 autopipets and 700-μl amber glass vial inserts were obtained from Rainin (Woburn, MA, U.S.A.). Limited-volume conical vial inserts (300 μl) and PTFE septa were purchased from Alltech Assoc. (Deerfield, IL, U.S.A.). Polypropylene syringes (3 ml) (Becton-Dickenson, Rutherford, NJ, U.S.A.) were used in conjunction with 0.45-μm, 13-mm Nylon syringe filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) for plasma filtration.

Preparation of spiked plasma samples

In order to prepare calibration curves and carry out spike recovery, accuracy, and precision studies, blank plasma was spiked with I and internal standard. Initially, six standards were prepared in water ranging from 0.02 to 10 μg/ml I each containing 1 μg/ml internal standard. Into conical 700-μl vials, 50 μl of standard solution and 450 μl of plasma were pipetted and vortex-mixed. The spiked plasma solutions were then filtered through nylon syringe filters into 300-μl conical vial inserts and capped with PTFE septa. The resulting spiked plasma solutions contained 2, 10, 30, 100, 300, and 1000 ng/ml I each with 100 ng/ml internal standard. All solutions were prepared daily from freshly thawed plasma and kept at 4°C during use.

Sample preparation procedure

Plasma samples were received frozen and stored at −70°C until analysis. Plasma samples were thawed, then 450 μl each pipetted into 700-μl conical vial inserts. Internal standard solution (50 μl) was then pipetted into each plasma sample and the solution vortex-mixed. The plasma was then filtered as described in *Preparation of spiked plasma samples.*

Chromatographic instrumentation and conditions

The HPLC system used two Waters Model 510 pumps, A and B (Waters Assoc., Milford, MA, U.S.A.), of which pump B was equipped with an SSI Lo-Pulse LP-21 pulse dampener (Scientific Systems, State College, PA, U.S.A.). The autosampler was a Waters WISP Model 712 equipped with a sample compartment refrigerator operated at 4°C (Waters Assoc.). The column-switching device was comprised of a Rheodyne Model 7000 six-port valve, Rheodyne Model 5701 pneumatic actuator, and a Rheodyne two-port, 12-V DC solenoid (Rheodyne, Westbury, NY, U.S.A.) which was controlled by two relays on a Waters System Interface Module (Waters Assoc.). The inline filter utilized a 0.5-μm stainless-steel frit (Upchurch Scientific, Oak Harbor, WA, U.S.A.). The drug concentration column was a Pinkerton ISRP glycine-phenylalanine-phenylalanine phase (GFF) guard cartridge, 5 μm particle size, 10 mm × 3 mm I.D. (Regis Chemical, Morton Grove, IL, U.S.A.). The analytical column was a Waters Radial Compression C_{18} cartridge, 4 μm, 10 cm × 5 mm I.D. which used a Waters 10 cm × 8 mm radial compression module which was kept at a constant 30°C temperature using a Waters temperature control module. The electrochemical detector utilized was an ESA Coulochem Model 5100A equipped with an ESA Model 5010 dual-electrode analytical module (electrode 1 set at +0.37 V, electrode 2 set at +0.6 V) and an ESA Model 5020 guard cell set at 0.8 V (ESA, Bedford, MA, U.S.A.). Strong mobile phase, solvent B, consisted of acetonitrile–0.02 *M* sodium phosphate, pH 7.5 (65:35). Weak mobile phase, solvent A, consisted of acetonitrile–0.02 *M* sodium phosphate, pH 7.5 (10:90). All mobile phase was filtered through a 0.22-μm Millipore GS membrane filter and degassed by helium sparging prior to use. Solvent B was pumped at a flow-rate of 1.5 ml/min (through C_{18} column), while solvent A was pumped at 1.0 ml/min (through ISRP column). Plasma samples were introduced onto the ISRP column using an injection volume of 100 μl unless otherwise noted.

Data acquisition and quantitation

Data collection and analysis was performed by a Microvax computer (Digital Equipment, Maynard, MA, U.S.A.) using Waters Expert Ease Chromatographic software (Waters Assoc.). The automated HPLC, electrochemical detector, and column switching valves were interfaced to the Microvax via a Waters system interface module (SIM) connected to a Waters laboratory acquisition and communication environment module (LAC/E).

RESULTS AND DISCUSSION

High-performance liquid chromatography

Chromatography of I on C_{18} supports had previously been extensively investigated by the authors and optimized for use in drug substance stability assays. The method incorporated a C_{18} radial compression cartridge, the internal standard II, and mobile phase B (strong mobile phase) described in the Experimental. This chromatographic method was then directly applied to the column-switchting plasma analysis method, functioning to remove retained substances from the ISRP support and carry out the analytical separation of I on a C_{18} column. Fig. 1 illustrates the structures of I, II, and an alternative internal standard III.

Initial methods development work involving the ISRP guard column was focused on defining a weak mobile phase which would retain I on this support. This work was performed without column switching by using only a pump, an autoinjector, and an ISRP guard column connected directly to a UV detector. It was

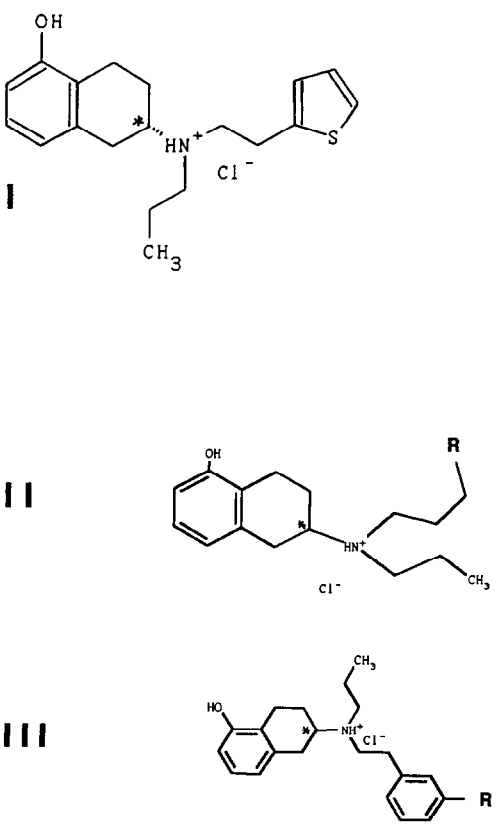

Fig. 1. Structures of (−)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride (I) and the internal standards, II and III, used in the study. The asterisk denotes asymmetric carbon atoms.

found that I was retained on the ISRP column for more than 20 min using mobile phase A (weak mobile phase). This indicated that mobile phase A would be suitable for concentrating I on the ISRP column. It was reasoned that if I was bound by proteins, the 10% acetonitrile in the weak mobile phase would help denature proteins and thus increase the recovery of the drug from plasma using the ISRP support. Since column switching was to be used in conjunction with electrochemical detection, efforts were made to keep the buffer concentrations and pH of the weak eluent the same as that used in the stronger mobile phase used to remove retained analytes and accomplish the analytical separation. It was observed that I was retained on the ISRP guard column for only 0.5 min using the strong mobile phase, indicating that this eluent could effectively remove the drug from the concentration column upon activating the column-switching valve.

Optimization of valve timing for column switching

Upon determining suitable mobile phases for use in the column-switching method, parameters involving timing of the valve switching required optimization. In particular, when the valve would: (1) switch to strong mobile phase, flushing retained substances from the ISRP support onto the C_{18} analytical column, and (2) switch back to weak mobile phase to re-equilibrate the ISRP column prior to the next injection.

Initially, the time required for protein removal from plasma using the ISRP guard cartridge was investigated. It was observed from the literature [35,36] that proteins appeared to elute rapidly from a 5 cm long ISRP support with drug retention times as small as 2.5–3.0 min using a flow-rate of 1 ml/min [35]. It was reasoned that using a much shorter ISRP guard cartridge would allow the proteins to be effectively removed in a shorter time, possibly on the order of 60–90 s. To test the time required for protein removal using the ISRP guard cartridge, the HPLC system was configured as depicted in Fig. 2, except that a UV detector was used. Repetitive 100-μl plasma injections were then made and proteins were allowed to elute under weak mobile phase conditions for 3 min before the valve switched to strong mobile phase. Between 30 and 50 injections could be made before a noticeable rise in back-pressure was observed for the analytical column, suggesting that a 3-min interval was sufficient time to remove most of the plasma proteins.

The last valve timing parameter required was the time at which the valve should switch weak mobile phase back to the ISRP support to allow the column to become re-equilibrated prior to the next injection. This required information on how quickly the strong mobile phase removed retained substances from the ISRP support. This was determined by using the column switching valve and injecting spiked plasma samples onto the ISRP support which was connected directly to a UV detector. Weak mobile phase was allowed to remove proteins for 3 min before the valve switched to strong mobile phase, while the UV detector output was monitored. It was observed that the strong mobile phase flushed all

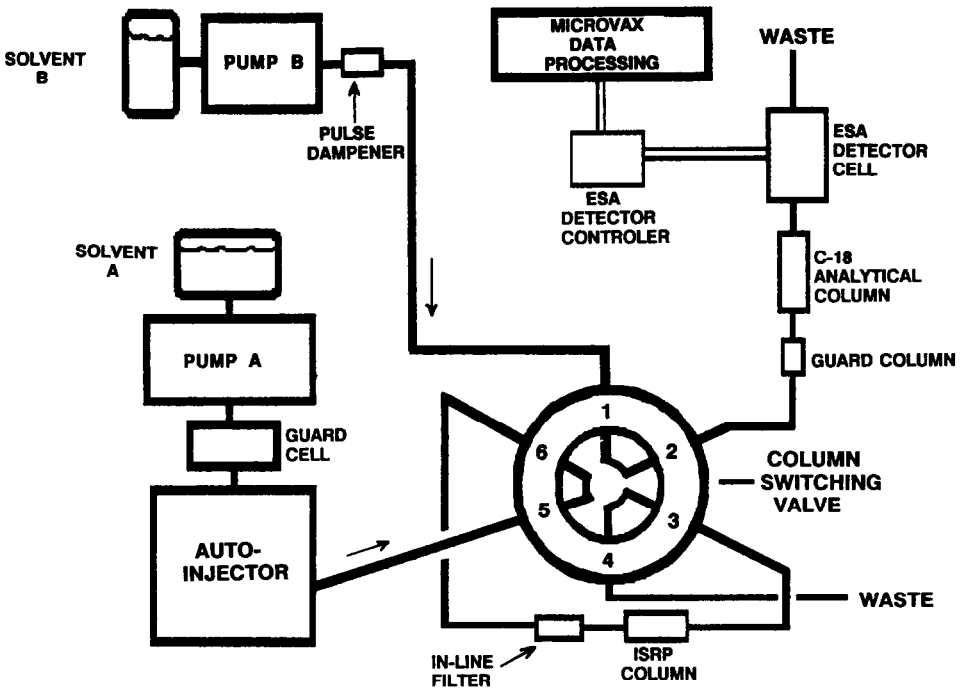

Fig. 2. Schematic diagram of the column-switching HPLC system used for analysis of I in plasma. Column-switching valve is shown in position A.

retained substances off the ISRP support within 15–20 s after the valve was switched, indicating that only a short flushing time was required.

Electrochemical detector optimization

The ESA electrochemical detector used in this work incorporated a dual-electrode detection cell which is designed to minimize interferences from other electroactive compounds. The detector cell consists of two porous graphite coulometric electrodes in series. Drug analyses and quantitation in this work was accomplished using the dual electrodes in the screen mode of detection, where the first electrode operates at a voltage slightly below the oxidation potential of I and acts to screen out electroactive interferences which are more easily oxidized. The second electrode, used for data acquisition, operates at a higher voltage which significantly oxidizes I, resulting in a chromatogram with potentially less interferences and background noise than conventional electrochemical detector designs.

In order to obtain the highest sensitivity possible for this assay, the optimum electrode potential of electrode 2 within the detector cell needed to be defined. The first step in determining this was to generate a hydrodynamic voltamogram (plot of I electrochemical response *versus* applied voltage). This was accomplished by assembling an HPLC system comprised of a pump, autoinjector, guard

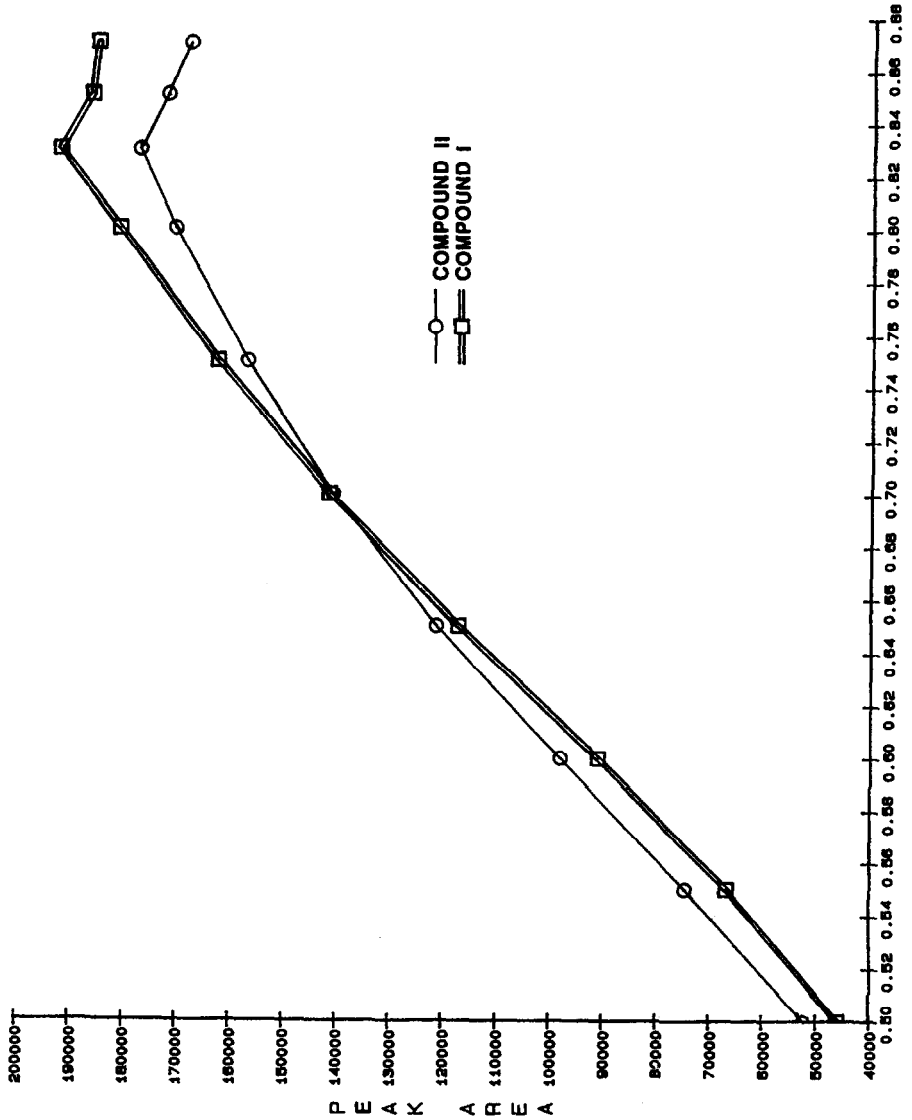

Fig. 3. Peak area of I and II plotted against the applied voltage of electrode 2 of the dual-electrode electrochemical detector (electrode 1 = 0 V).

column, and electrochemical detector cell. After equilibrating the system with strong mobile phase, an aqueous 100 ng/ml standard of I was then repeatedly injected as the potential was increased in 0.05-V increments (electrode 1 = 0 V). This potential was increased until the I peak height remained constant. The resulting plot of voltage *versus* peak height for I and II is shown in Fig. 3 where it can be seen that both electrochemical responses level off after 0.83 V. We chose the lower working potential of 0.65 V for the method since it was found that the background signal and noise increased exponentially above 0.65 V, causing lower signal-to-noise ratios. Also, more interferences from endogenous plasma components were seen at higher potentials. We found that the 0.65-V potential offered the optimum signal-to-noise ratio for the analysis as well as extending the lifetime of the detector cell.

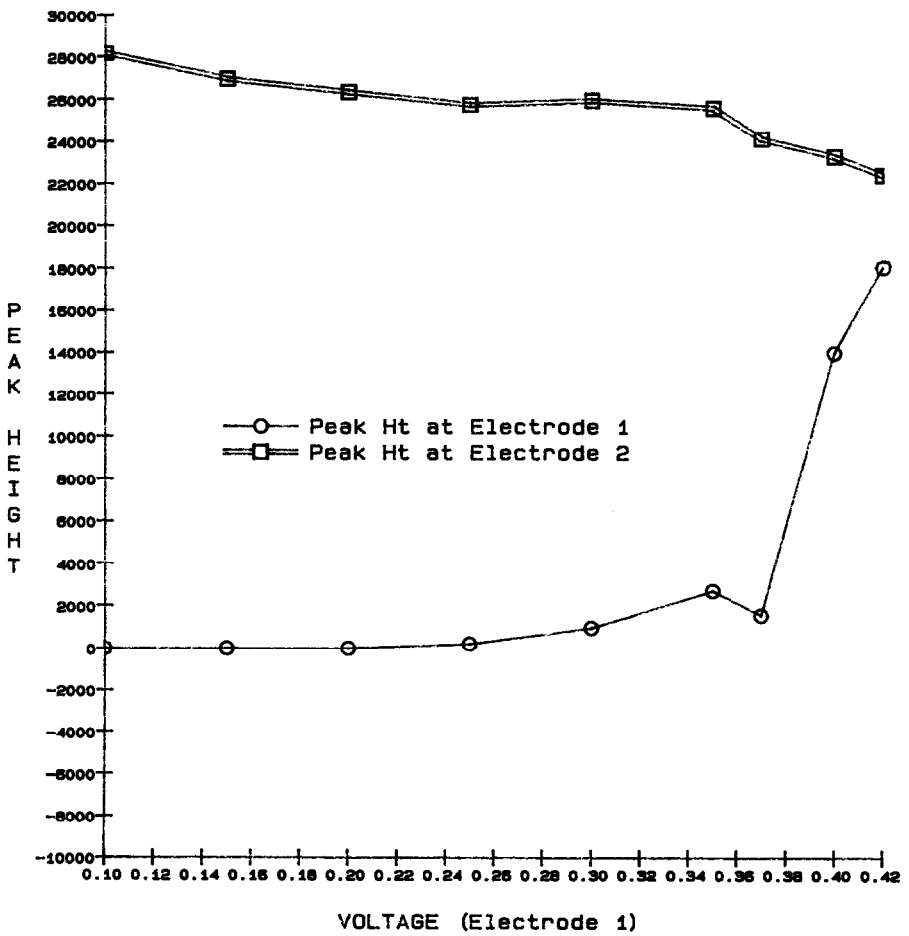

Fig. 4. Peak height of I at electrodes 1 and 2 plotted against the applied voltage of electrode 1 of the dual-electrode electrochemical detector (electrode 2 = 0.65 V).

Optimization of the detector for screen mode of analysis required electrode 1 of the detector to be operated at the highest voltage possible without oxidizing I. This first electrode would then help minimize peaks due to interfering plasma components. Optimization of the potential of electrode 1 was determined by the same method as for electrode 2, except the voltage of electrode 1 was varied while electrode 2 was held constant at 0.65 V. The peak height of I was then recorded at both electrodes 1 and 2. The resulting data are plotted in Fig. 4. It can be seen that response of I at electrode 2 does not decrease until electrode 1 reaches a potential of 0.37 V. From this plot it was concluded that 0.37 V was the optimum potential at which to operate electrode 1 of the detector.

HPLC column-switching apparatus

A diagram of how the column-switching HPLC system was configured is shown in Fig. 2. The two pumps are connected to the Rheodyne 7000 valve such that with the valve in position A (as in Figure 2), weak mobile phase passes through the autosampler, electrochemical guard cell, in-line filter, ISRP column, and finally to waste. The electrochemical guard cell is operated at a voltage higher than that of the analytical cell electrodes and is designed to scrub out electroactive background interferences in the weak mobile phase which would otherwise be concentrated on the ISRP column. With the valve in position A, the analysis starting position, drugs are concentrated on the ISRP column and proteins eluted to waste. The valve in this position also directs the strong mobile phase through a pulse dampener, a guard and analytical column, then finally through the electrochemical cell. After a 100-μl injection of plasma, 3 min elapse while plasma proteins elute, then the valve is automatically switched to position B which allows strong mobile phase to flow through the ISRP support, stripping retained drug and internal standard into the analytical column where they are separated and detected. The valve automatically switches back to position A at 4 min to allow the ISRP support to equilibrate with weak mobile phase prior to the next injecton.

Plasma analysis using column switching

After identifying suitable mobile phases, determining the times at which the column switching valve should actuate, and optimizing the electrochemical detector parameters, the system was assembled as shown in Fig. 2 and spiked plasma samples were analyzed. Fig. 5A and B show the chromatograms obtained for blank bovine plasma and bovine plasma spiked with 100 ng/ml each of I and II, respectively. Fig. 5C shows monkey plasma measured at 95 ng/ml after I was administered intravenously. After linearity, accuracy and precision of the method were demonstrated, plasma from toxicological and pharmacokinetic studies were analyzed. The chromatograms in Fig. 5 are typical to many of those obtained for the various *in vivo* experiments using I in rats and monkeys.

During the course of analyzing numerous plasma samples using the column-

switching technique, we found that endogenous plasma components, anesthetics, etc. occasionally interfered with the integration of the internal standard II. When this occurred, we found it advantageous to be able to substitute a different in-

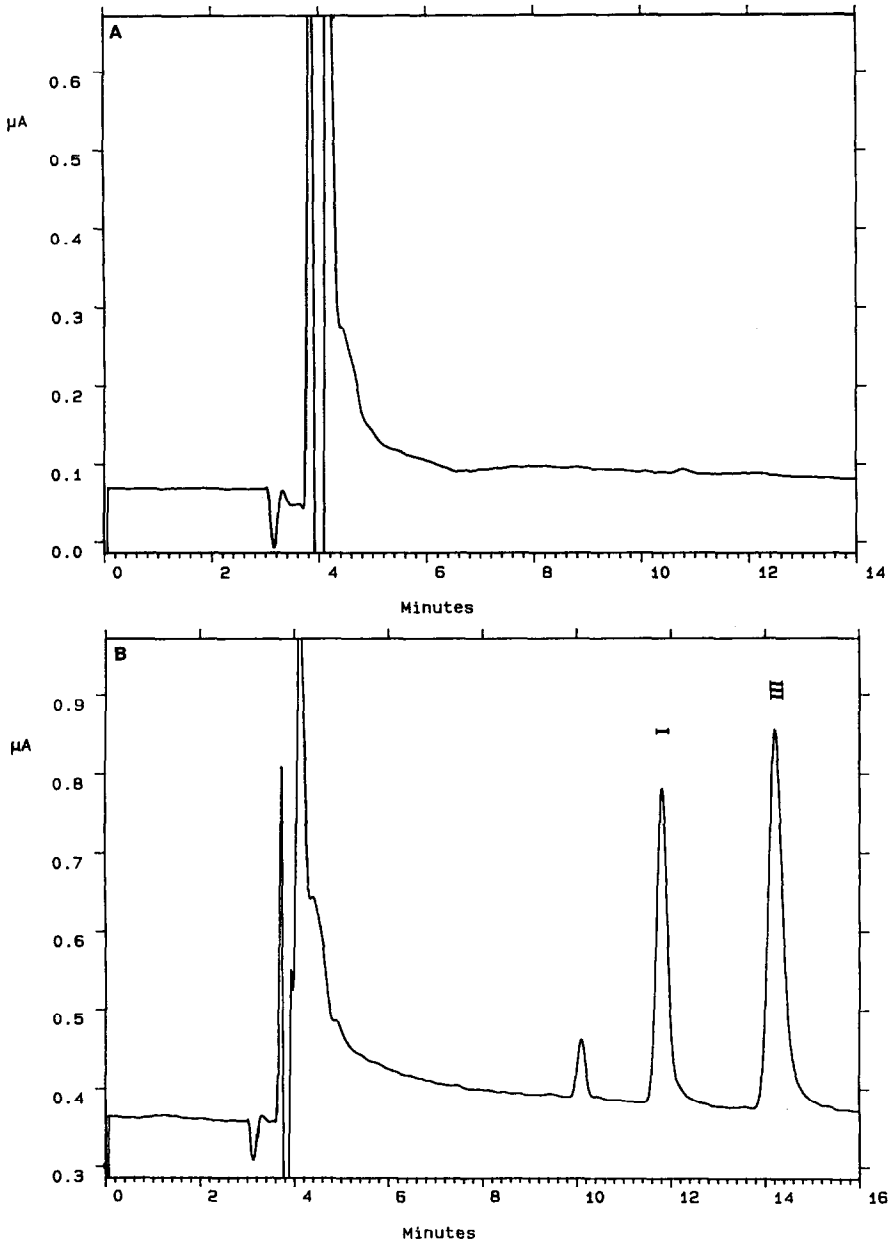

Fig. 5.

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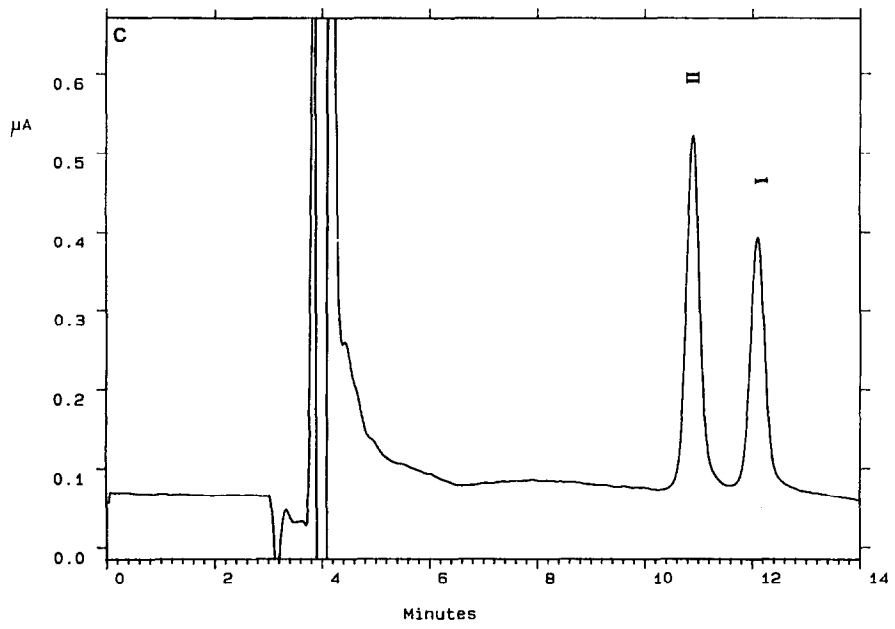

Fig. 5. Typical chromatograms obtained from analysis of I in plasma using column switching with electrochemical detection. (A) Blank bovine plasma; (B) bovine plasma spiked with 100 ng/ml each of I and II; (C) plasma from monkey which received intravenous injection of I. Measured concentration = 95 ng/ml I.

ternal standard (Fig. 1) of a different retention time, allowing more accurate quantitation of I in many cases.

The column-switching method was ideally suited to large numbers of samples since sample work-up consisted only of plasma filtration and addition of internal standard. The minimal sample handling of the method introduced fewer interferences compared to solid phase extraction (SPE) procedures [34] which were previously performed by the authors. The method of direct injection of plasma is very advantageous when the sample volume is limited, as the sample work-up can be modified to accommodate as little as 100 μl of plasma. The method is rapid, completely automated, and significantly reduced the time and manpower required compared to the SPE method [34].

The method performed well throughout the analysis of over 500 plasma samples from various *in vivo* pharmacokinetic and toxicological animal experiments. As a precautionary measure, the ISRP and guard columns were replaced after every 40 plasma injections so rising back-pressure would be avoided. Detector cell sensitivity was observed to slowly diminish after approximately 100–200 plasma injections, but was returned to normal in most cases by flushing with acetonitrile or 4 *M* nitric acid.

Drug recovery from plasma

Drug recovery from plasma was calculated by preparing spiked bovine samples as described in *Preparation of spiked plasma samples*. These samples were then analyzed using the column-switching method and compound I concentrations for the spiked plasma solutions quantitated using analogous aqueous calibration standards. The recovery values obtained are shown in Fig. 6, which clearly indicates that recovery of I from plasma using the ISRP column is concentration-dependent. One explanation for the variation in recovery is that I may be strongly bound to plasma proteins and albumin. The binding characteristics of the drug may then become more apparent at low concentrations because incomplete protein denaturation may reduce the amount of drug interacting with the inner surfaces of the ISRP support. The dependence of recovery on concentration was observed to also vary slightly between the different rat, bovine, monkey, and human plasma samples. Other factors which may influence recovery are the short ISRP column length, the weak organic content of mobile phase A, and the large plasma injection volumes used.

In an effort to determine if injection of larger volumes of plasma would increase the sensitivity of the assay, various volumes of 100 ng/ml spiked bovine plasma were injected onto the ISRP column. Percentage recovery of I from plasma was calculated by comparing I peak areas to peak areas of identical injections of aqueous 100 ng/ml standards. It can be seen in Fig. 7 that the I recovery from the ISRP guard cartridge is also dependent upon plasma injection volume. It is probable that large volumes of plasma overload the ISRP support, causing the drug to be carried through to waste with proteins. It was concluded that the 100-μl injection volume typically used in the assay offered the desired sensitivity with acceptable recovery.

Assay linearity

Since recovery of I varied with concentration as well as the type of plasma, it was concluded that the most accurate method of quantitation would be achieved by using spiked plasma solutions as calibration standards rather than aqueous standards. Since recovery also varied slightly with different types of plasma, blank plasma of the same animal species as the unknown samples was used for preparation of the calibration standards. This helped compensate not only for the general variation in recovery with concentration, but also for the recovery variation due to different plasma types. Due to the limited blood volume involved in rat studies, blank plasma was not available for use in preparing calibration standards, and bovine plasma was substituted.

Linear response was observed from 1.5 to 1000 ng/ml when using either internal standard in the analysis of rat, monkey and human plasma. Typical correlation coefficients ranged from 0.9990 to 0.9998. Fig. 8 shows a chromatogram of spiked human plasma at the detection limit of the assay which was 1.5 ng/ml.

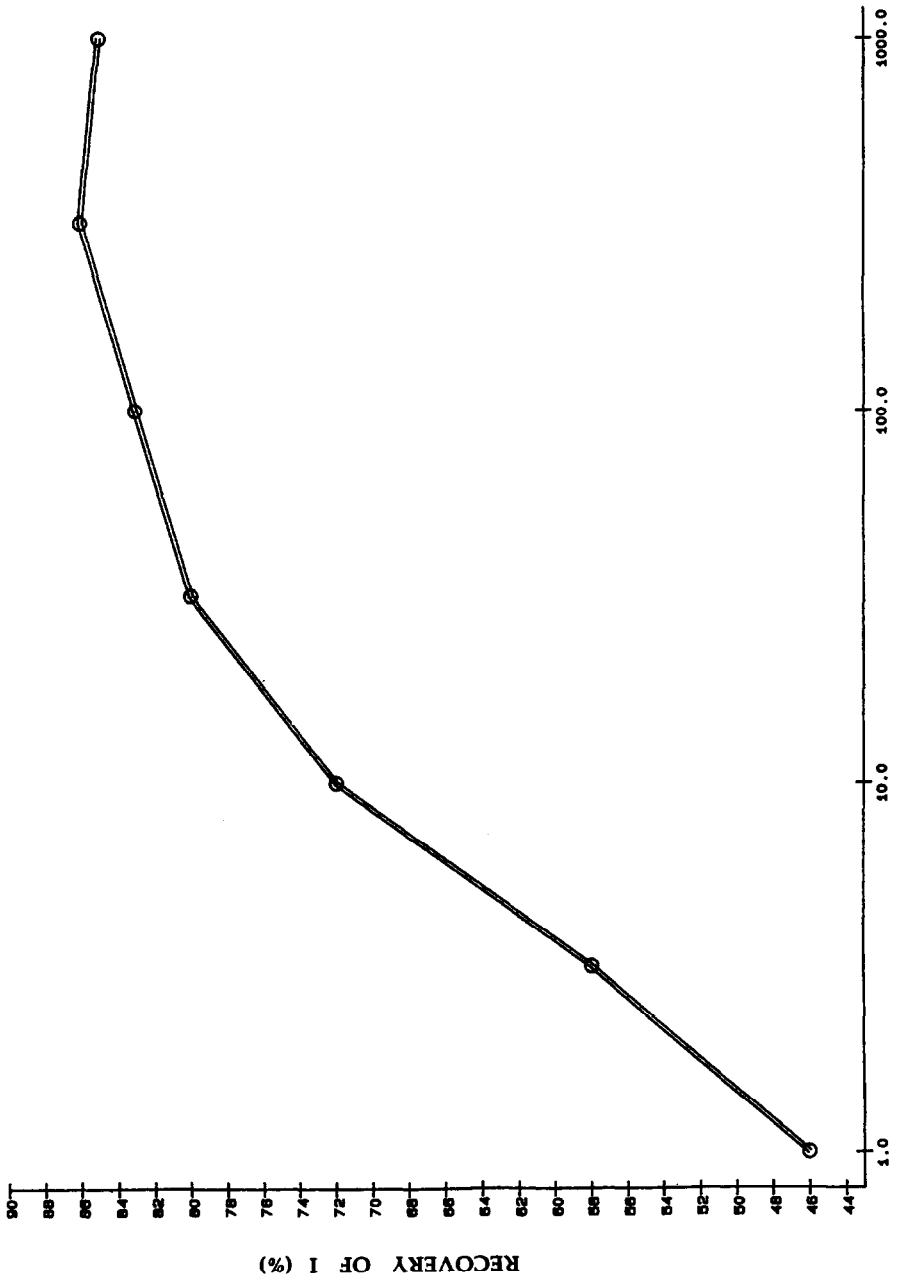

Fig. 6. Recovery of I from plasma as a function of sample concentration.

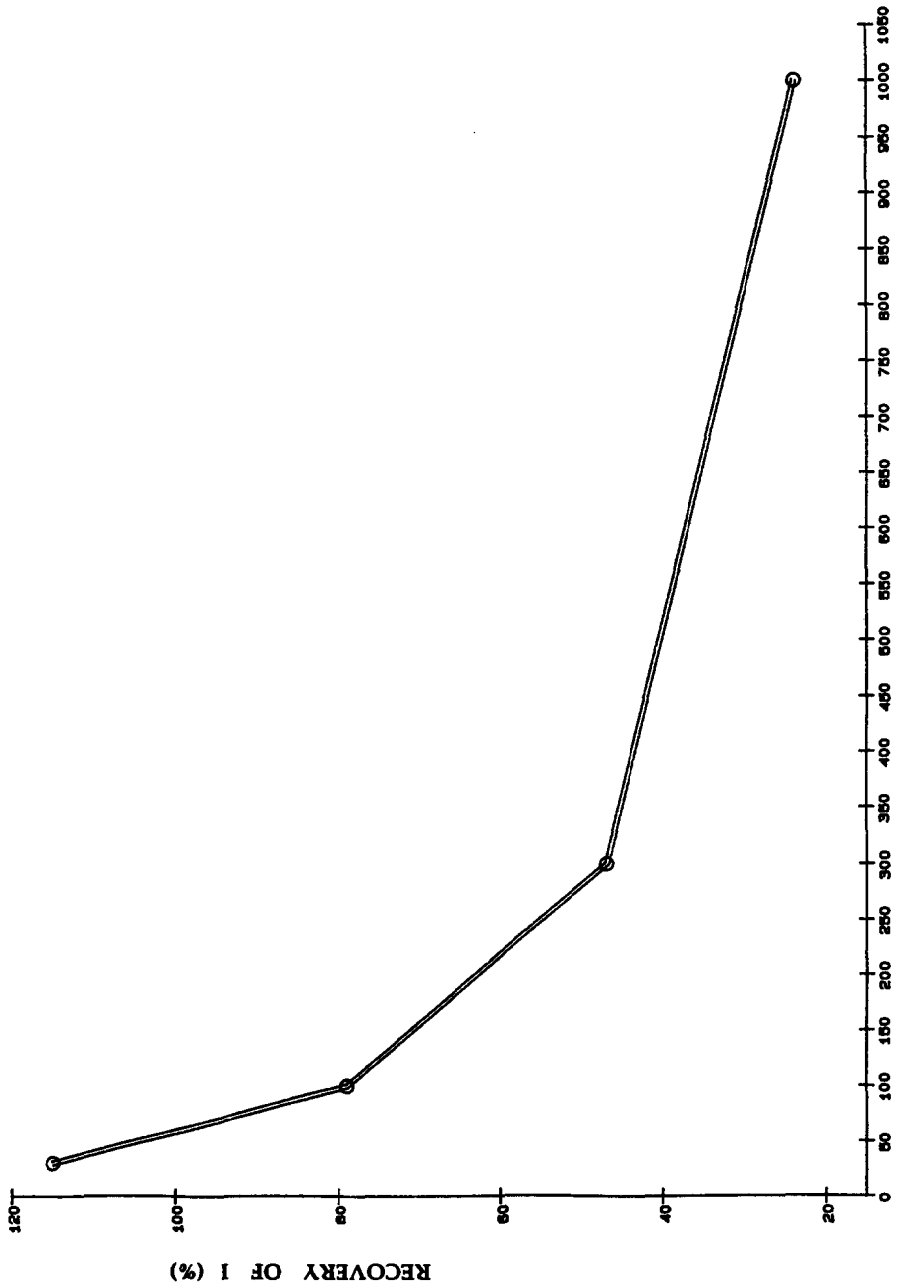

Fig. 7. Recovery of I from plasma as a function of plasma injection volume.

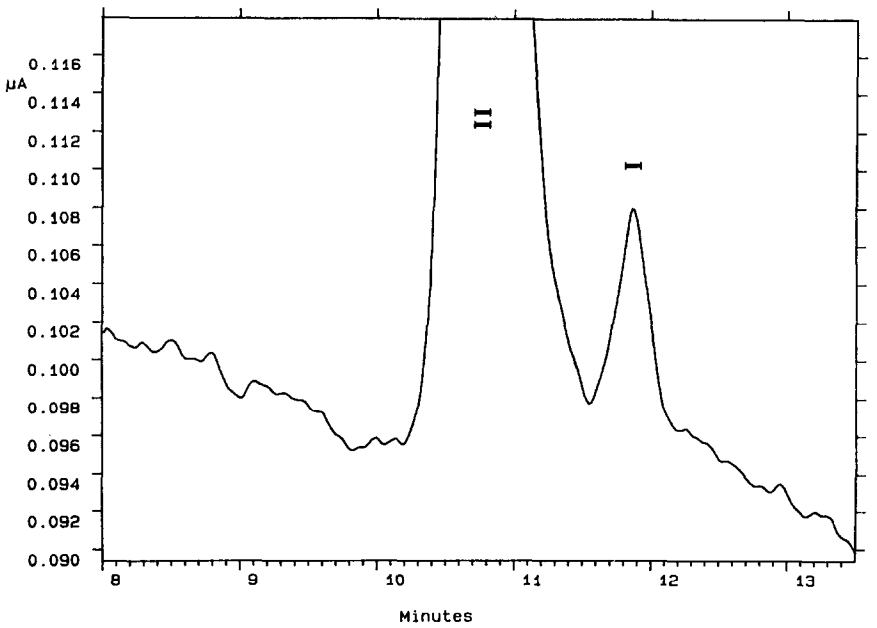

Fig. 8. Chromatogram of spiked human plasma at the detection limit of the method, 1.5 ng/ml I.

Assay precision and accuracy

Precision of the assay was determined by preparing spiked bovine plasma as described in *Preparation of spiked plasma samples*. These plasma solutions were then injected in triplicate and the relative standard deviation (R.S.D.) of I peak area as well as response *versus* internal standard calculated. The results appear in Table I where it can be seen that a maximum R.S.D. of 4.7% is obtained for I response *versus* internal standard. An additional precision experiment was car-

TABLE I

PRECISION DATA FOR COLUMN-SWITCHING METHOD

Spiking level of I (ng/ml)	R.S.D. of peak area (n = 3) (%)	R.S.D. for peak area of I/internal standard[a] peak area (n = 3) (%)
2.5	11.6	4.7
10.1	2.9	3.6
30.3	2.3	2.5
101.3	2.3	1.4
304.0	1.5	0.1
1013.1	0.6	0.3

[a] Internal standard = compound II.

TABLE II

ACCURACY DATA FOR COLUMN SWITCHING METHOD ($n = 3$)

Spiking level of I (ng/ml)	Concentration found (ng/ml)	Percentage of theory[a]
1.0	0.4	40
10.0	11.5	115
14.5	14.3	99
30.0	30.6	102
101.3	108.0	107
1013.0	1076.0	106

[a] Percentage of theory = (spiking level of I/concentration found) × 100.

ried out by preparing a 100 ng/ml spiked bovine plasma solution, making six aliquots, working up each aliquot with internal standard, then making duplicate injections of each solution. The R.S.D for response factors was 4.4%.

Accuracy was determined by preparing spiked human plasma samples at 1, 10, 14, 30, 100, and 1000 ng/ml. Emphasis was placed in the 1–100 ng/ml range because accuracy and recovery were most variable at lower concentrations. The spiked plasma samples were then analyzed using spiked human plasma as calibration standards. The percentage of theory [(true value/calculated value) × 100] was then calculated and appears in Table II. Accuracy was lower than desired at the 1 ng/ml level, however, because this is at the detection limit of the assay, the 35% of theory value was considered acceptable. All other concentrations show good accuracy, although slightly above 100% of the true concentration. Although there is more error in the 1–10 ng/ml range, additional standards in this range were not used because a majority of the pharmacokinetic and toxicological plasma samples analyzed yielded concentrations of I above 10 ng/ml.

TABLE III

REPRODUCIBILITY OF COLUMN-SWITCHING METHOD AT 20 ng/ml

Operator	n	Day	Amount of compound I found (ng/ml)	R.S.D. (%)
A	6	1	21.3	5.2
A	6	6	21.5	6.3
B	5	7	20.6	4.7
Mean			20.6	6.5

Assay reproducibility

Bovine plasma was spiked at a concentration of 20 ng/ml and the sample analyzed by two operators on different days to determine the reproducibility of the method. On each day spiked bovine calibration standards were prepared as described in *Preparation of spiked plasma samples*. The results appear in Table III from which it can be seen that the assay yields a person-to-person, day-to-day variation of 6.5% at the 20 ng/ml level.

CONCLUSION

The applicability of a two-dimensional column-switching HPLC method using direct plasma injection to accurately measure I was demonstrated. The method is reproducible and exhibits satisfactory precision and accuracy. The method is sensitive and rapid, requiring a total analysis time of 20 min per sample, and has a 1.5 ng/ml limit of detection. The method was successfully applied to human plasma as well as large numbers of samples from pharmacokinetic and toxicological studies of I in rats and monkeys.

The type of analytical methodology presented could be applied to a variety of other drugs because the two-dimensional nature of the technique allows significant flexibility in choosing stationary phases to accomplish the analytical separation. This allows application of the technique to a wide range of analytes, assuming that they can be retained by the ISRP support and subsequently detected. A major advantage of this method when applied to biological samples is that very small sample volumes can be directly analyzed, which is especially useful in rodent and small animal studies.

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