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ANALYSIS OF RIBOXAMIDE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN SWITCHING

C.M. RILEY, L.A. STERNSON* and A.J. REPTA

Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045 (U.S.A.)

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SUMMARY

A sensitive and highly specific assay for riboxamide (TCAR) in human and canine plasma is described. The specificity of the procedure is derived from the method of sample preparation and a high-performance liquid chromatographic separation which utilizes the different selectivities of two columns. Partial separation of TCAR from plasma is achieved on a solvent-generated anion exchanger with silica gel as the solid support. The separation is completed by switching the eluent fraction containing TCAR from the first column to a second solvent-generated anion exchanger which has ODS-silica as its support. The relationship between the amount of drug injected and its peak height was linear over wide ranges of concentrations (0—10 μg/ml) and injection volumes (20—200 μl). The limit of detection for TCAR in plasma was 40 ng/ml which can be detected by injecting 200 μl of processed plasma. The recoveries from plasma were 100.2 ± 0.9% and 101.3 ± 2.3% when spiked at the 10 and 1 μg/ml levels, respectively. The applicability of the method to pharmacokinetic studies was demonstrated by following the plasma levels of TCAR after intravenous administration in the dog.

INTRODUCTION

Riboxamide (TCAR, 2-β-D-ribofuranosyl-4-thiazolcarboxamide, NSC 286193) is a C-nucleoside (Fig. 1) which was synthesized originally [1, 2] as an analogue of ribavirin. Despite exhibiting significant activity against rhino-, influenza and herpes viruses [2], the potency of TCAR was less than that of ribavirin [2]. The initial observation [2] that TCAR is a potent inhibitor of guanine synthesis led to it being investigated as an antineoplastic agent [3, 4]. It was found to have significant activity against L1210 and P388 leukemias and Lewis lung carcinoma, both in vitro [3] and in vivo [4]. As a result of these studies, TCAR is now undergoing (Phase 1) clinical trials.

High-performance liquid chromatography (HPLC) has been used to separate

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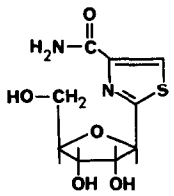

Fig. 1. The structure of riboxamide (TCAR).

the anionic metabolites of TCAR [3, 4], however, the drug itself is poorly retained on the strong anion-exchange column (Partisil 10 SAX) used. Presently, there are no analytical procedures which would be suitable for studying the pharmacokinetics of TCAR, and the development of such methodology is the subject of this investigation.

EXPERIMENTAL

Chemicals and reagents

The sample of TCAR was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Hexadecyltrimethylammonium bromide (HTAB) was 99% pure from Aldrich (Milwaukee, WI, U.S.A.). All the other chemicals (chloroform, Na_2HPO_4, KH_2PO_4, sulfuric acid, perchloric acid and sodium hydroxide) were analytical grade from various sources. The water was distilled-in-glass, following mixed-bed deionization.

Plasma preparation

Plasma (human or canine) samples were prepared for analysis as described in Fig. 2. The plasma proteins were precipitated with perchloric acid [5] and the supernatant, obtained after centrifugation (1500 *g*), was extracted twice with chloroform. The aqueous phase was then made alkaline (pH ca. 11.5) with sodium hydroxide (10 *N*) and re-extracted twice with chloroform. HTAB was added to the recovered aqueous phase to give a final concentration of 1 m*M* HTAB. The precipitate which was formed was then removed by centrifugation (7000 *g*), and the final aqueous supernatant was analyzed by HPLC. It should be noted that the precipitate formed upon the addition of HTAB will start to resuspend if the supernatant is not removed immediately.

Chromatography

The liquid chromatography (Fig. 3) was built from various modules and designed so that selected fractions eluting from column 1 could be transferred to column 2. The system was fully automated, controlled by SLIC 1400 microprocessor (Systec, New Brighton, MN, U.S.A.) and consisted of two Altex 152 detectors (Beckman Instruments, Berkeley, CA, U.S.A.) operated at 254 nm; a WISP 710 automatic injector (Waters Assoc., Milford, MA, U.S.A.); two Altex 110A pumps; a four-port, low-pressure, switching valve (valve 1) and a Rheodyne 730 valve (valve 2, Cotati, CA, U.S.A.).

The μBondapak CN (particle size 10 μm, 300 × 3.9 mm I.D.) and Partisil 10 SAX (particle size 10 μm, 250 × 4.6 mm I.D.) columns were obtained from Waters Assoc. and Whatman (Clifton, NJ, U.S.A.), respectively. The

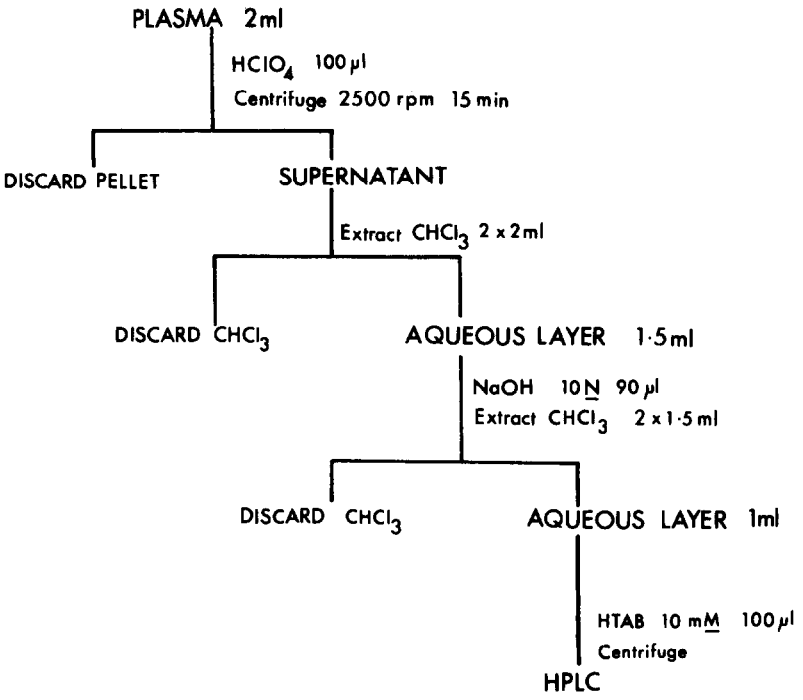

Fig. 2. Summary of the preparation of plasma samples for analysis by HPLC.

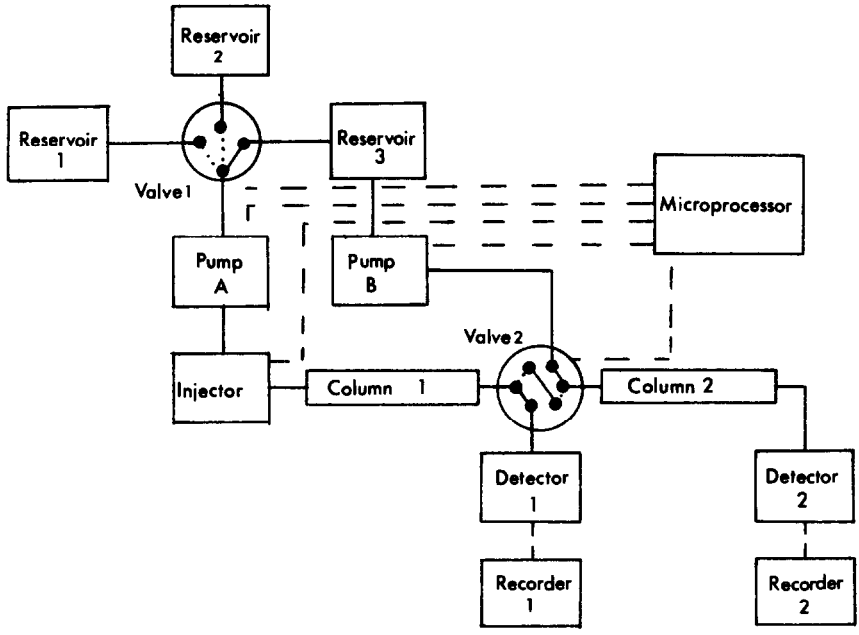

Fig. 3. Schematic representation of the chromatographic system assembled for the analysis of TCAR in plasma. See text and Fig. 4 for further explanation.

Hypersil (particle size 5 μm, 150 × 4.6 mm I.D.), ODS Hypersil (particle size 5 μm, 150 × 4.6 mm I.D.) and μBondapak C_{18} (particle size 10 μm, 250 × 4.6 mm I.D.) columns were slurry packed, as described previously [6, 7]. The Hypersil and μBondapak bulk packings were obtained from HETP (Macclesfield, Great Britain) and Waters Assoc., respectively.

For the analysis of TCAR in plasma (Figs. 3 and 4), columns 1 and 2 were packed with Hypersil and ODS Hypersil (or μBondapak C_{18}), respectively.

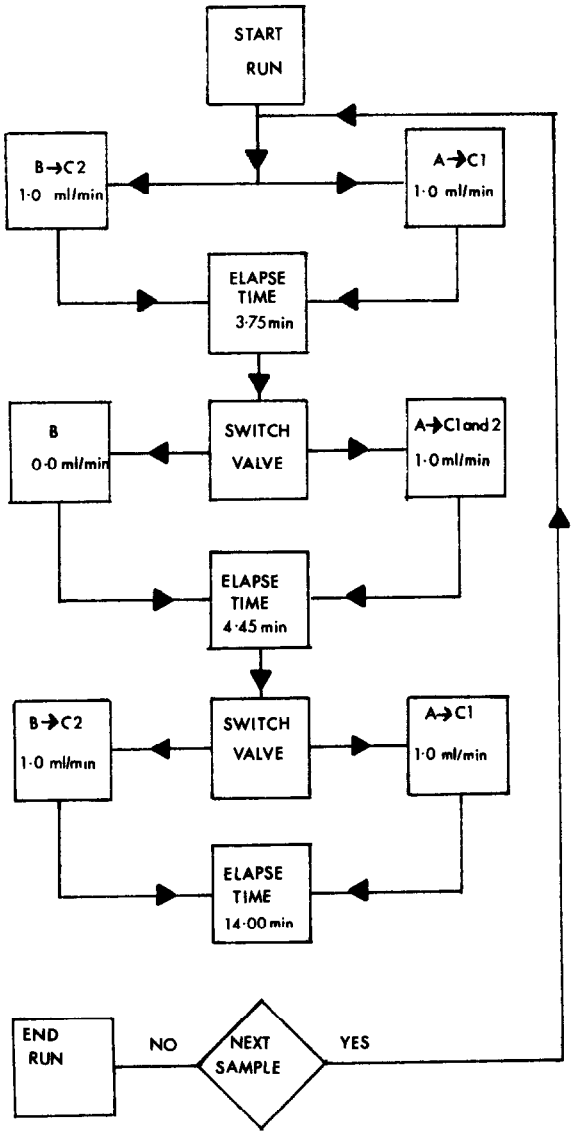

Fig. 4. Summary of the switching events for the analysis of TCAR in plasma. A, B, C1 and C2 refer to pumps A and B, column 1 (Hypersil) and column 2 (ODS Hypersil or μBondapak C_{18}), respectively. The arrows (→) within the boxes indicate the flow from the respective pumps through the columns. The elapse times are from the point of injection. See Fig. 3 for further details.

Both columns were eluted with a 10 m*M* phosphate buffer (pH 6.0) containing 1 m*M* HTAB. After injection of the sample, the fraction containing TCAR (800 μl) which eluted from column 1 was transferred to column 2, where the separation was completed. The specific details of the switching events are summarized in Fig. 4.

To remove components of the plasma preparation which failed to elute from column 1, this column was purged after each working day with water (50 ml), followed by aqueous solutions of increasing methanol concentration added in 20% increments (50 ml of each) up to 40% methanol. This procedure was facilitated by the low-pressure switching valve positioned between pump A and the three reservoirs (Fig. 3). It was not necessary to routinely wash column 2 since its chromatographic properties remained constant.

The pharmacokinetics of TCAR in the dog

TCAR (25 mg/kg) was administered to a female Beagle dog (10 kg) by injection (1 ml) into the saphenous vein. Prior to administration of the drug, the dog was fasted overnight and a "baseline" blood sample taken from the jugular vein. Blood samples (2 ml) were taken 2, 5, 15, 60, 120, 180, 270 and 360 min after administration and stored in glass vials containing EDTA. The erythrocytes were removed by centrifugation (1200 *g*, 15 min) and the plasma analyzed for TCAR, as described above.

RESULTS AND DISCUSSION

Selectivity

Analytical selectivity was achieved by the combination of sample preparation and chromatographic separation. Initially, single column systems using reversed-phase or anion-exchange stationary phases were investigated for the separation of TCAR from plasma (Table I). The drug was only adequately retained on two of the four reversed-phase columns (Table I), even with purely

TABLE I

RETENTION OF RIBOXAMIDE IN VARIOUS CHROMATOGRAPHIC SYSTEMS

Column	Mobile phase*	k'
Reversed-phase		
ODS Hypersil	A	6.20
	B	6.18
μBondapak C_{18}	B	4.16
μBondapak CN	B	0.10
Hypersil	B	0.00
Anion exchange		
Partisil SAX	B	0.10
μBondapak C_{18} + HTAB	C	1.93
ODS Hypersil + HTAB	C	1.99
Hypersil + HTAB	C	1.10

*Mobile phases: A = 0.1% sulfuric acid (pH 2.1); B = 10 m*M* phosphate buffer (pH 7.0); C = 10 m*M* phosphate buffer (pH 6.0) and 1 m*M* HTAB.

aqueous mobile phases. The highest retention was observed on ODS Hypersil ($k' = (t_R - t_0)/t_0 = 6.2$). The retention of TCAR on all the columns studied was independent of mobile phase pH (2—7), suggesting that its ionization state did not change over this range.

The lack of anionic groups on TCAR accounts for its poor retention on Partisil 10 SAX [3, 4], however, significant retention on solvent-generated anion exchangers was observed. Such columns were prepared by adsorbing a monolayer of HTAB onto the surface of silica (Hypersil) and ODS-silica (ODS Hypersil and μBondapak C_{18}) columns [8—10]. Solutions containing 10 m*M* HTAB were used to coat the columns with surfactant and the stability of the modified stationary phases was maintained, subsequently, by the presence of 1 m*M* HTAB in the mobile phase.

The retention of neutral drugs (platinum complexes) on solvent-generated anion exchangers has been observed previously [8—10], and attributed to ion—dipole interactions between the solutes and the cationic surfactant (HTAB) adsorbed onto the stationary phase. In the case of TCAR, however, these ion—dipole interactions are probably less important since the drug is retained less on ODS Hypersil and μBondapak C_{18} in the presence of HTAB than in its

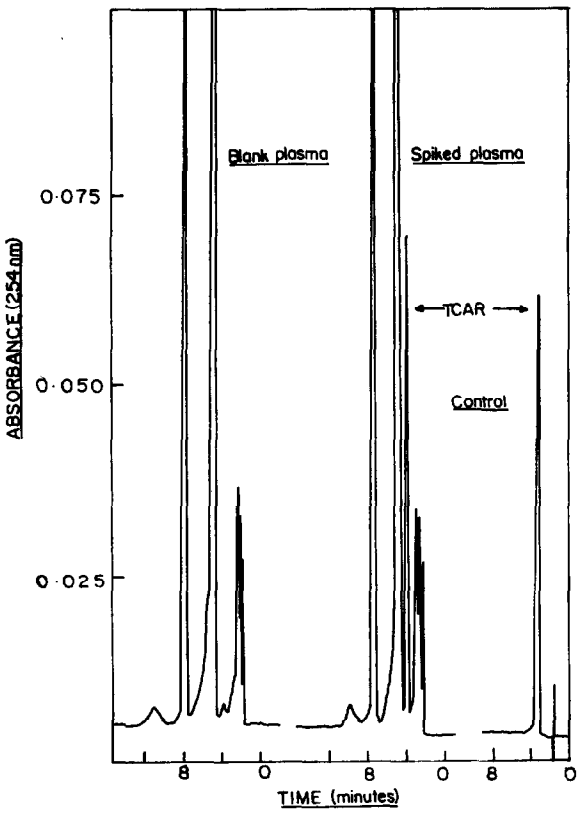

Fig. 5. Chromatogram showing the partial resolution from human plasma on ODS Hypersil, modified by the adsorption of HTAB onto its surface. Mobile phase: 10 m*M* phosphate buffer (pH 6.0) containing 1 m*M* HTAB; flow-rate: 1.0 ml/min; temperature: ambient; TCAR concentration: ca. 10 μg/ml.

absence (Table I). Similar decreased retention of neutral molecules on reversed-phase columns has been reported [11] and attributed to a reduction in the hydrocarbonaceous surface area of the stationary phase, produced by the adsorption of charged ions from the mobile phase. In contrast with ODS-silica columns, adsorption of HTAB onto the surface of silica (Hypersil) apparently enhances the hydrophobic character of the stationary phase, causing the observed increased retention of TCAR compared with that measured on bare silica (Table I).

Both reversed-phase and anion-exchange columns were investigated for the separation of TCAR from plasma, using a variety of aqueous mobile phases (pH 2—7). The weak eluents required for adequate retention of TCAR also resulted in significant retention of plasma components which interfered with TCAR. Although some of the interfering plasma components could be removed by extraction into chloroform (see Experimental), the use of two columns with different selectivities was required to achieve adequate resolution. In all cases using a single column, the TCAR peak was heterogeneous, as established subsequently by column switching techniques.

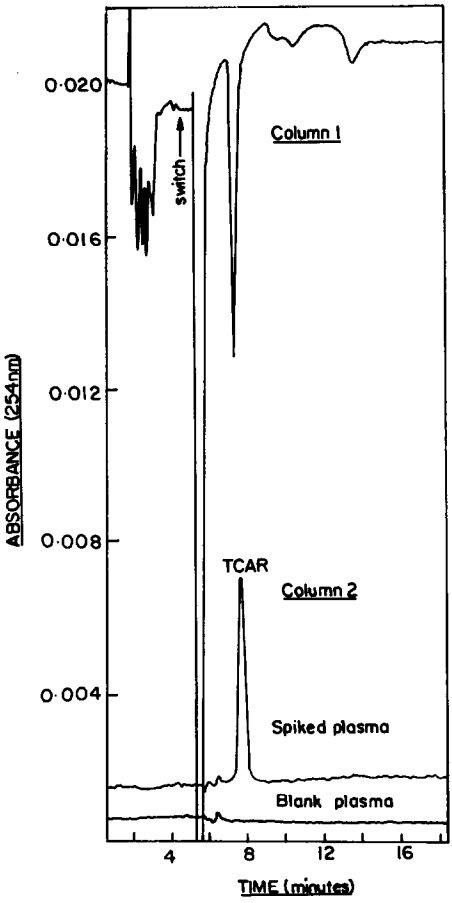

Fig. 6. Chromatogram showing the complete separation of TCAR from human plasma using column switching. See text and Figs. 3—5 for chromatographic conditions.

Optimal resolution of TCAR from plasma (Figs. 5 and 6) was achieved by partial separation on silica (Hypersil) coated with HTAB (Fig. 5); followed by transfer of the fraction containing the analyte to an ODS-silica column (ODS Hypersil or μBondapak C_{18}) coated with HTAB. It was necessary to use the same mobile phase for the elution of both columns, otherwise displacement peaks interfered with TCAR. The optimum mobile phase was 10 m*M* phosphate buffer (pH 6.0) containing 1 m*M* HTAB.

Precision and linearity

The peak height of TCAR was linearly related to the amount of solute (*q* in ng) injected, according to eqn. 1:

$$P = a \cdot q + b \quad (1)$$

where *a* and *b* are the slope and intercept of the linear regression (see Table II). The peak heights (*P* in mm) were corrected for changes in detector attenuation, using 0.02 a.u.f.s. as the reference. The response factor (*a*) was independent of detector attenuation (0.005—0.160), but decreased slightly with increasing injection volume (20—200 μl). This was attributed to a slight increase in band broadening with increasing volume of injection.

TABLE II

VARIATION OF RESPONSE FACTOR (*a*) WITH INJECTION VOLUME

Chromatographic conditions			Regression analysis*		
Injection volume (μl)	Concentration** range (μg/ml)	Detector*** setting (a.u.f.s.)	*a*	*b*	*r*
20	0—10	0.020	0.70	—0.26	0.9996
	0—2	0.005	0.69	—0.30	0.9993
50	0—10	0.040	0.67	—0.07	0.9998
	0—2	0.010	0.67	0.03	0.9999
100	0—10	0.080	0.65	—3.02	0.9995
	0—2	0.020	0.66	—0.21	0.9998
	0—0.2	0.005	0.64	—0.19	0.9969
200	0—10	0.160	0.62	—0.88	0.9996
	0—2	0.040	0.63	1.67	0.9998
	0—0.2	0.005	0.59	—0.02	0.9997

*Eqn. 1.

**n = 6 in all cases (samples prepared contained 0, 20, 40, 60, 80 and 100% of the concentration in each range studied).

***These represent the most appropriate detector attenuations for the analysis of TCAR in the stated ranges of concentration and injection volumes.

The day-to-day reproducibility (expressed as coefficient of variation, $n \geq 4$) of the response factor was 5.8%. Consequently, the response factor was checked by injection of an external standard, after every fourth plasma sample, using the same injection volume that was used for the samples. Within a single day, the coefficient of variation of the peak heights of the external standards was less than 0.7% (n = 6) for injections of 20—200 μl. The larger day-to-day

variation in response factor was probably due to fluctuations in room temperature and/or slight changes in the chromatographic properties of column 1.

Although ODS Hypersil and μBondapak C_{18}, coated with HTAB, gave adequate resolution when used as column 2, the shorter ODS Hypersil column was preferred since it offered shorter analysis times. With ODS Hypersil as column 2, the analysis time (14 min) was limited by the time required to elute the remaining plasma components from column 1. Sensitivity was not compromised by replacing μBondapak C_{18} with ODS Hypersil, since they gave very similar response factors (a = 0.73 ± 0.02 S.E.M. for ODS Hypersil and 0.70 ± 0.002 S.E.M. for μBondapak C_{18}). The limit of detection for TCAR in plasma was 40 ng/ml which gave a signal-to-noise ratio of 4:1 with an injection of 200 μl.

Recovery and compatibility of the plasma extract

After correction for dilution, the recovery of TCAR from plasma (Fig. 2) was 100.2 ± 0.9% S.E.M. and 101.3 ± 2.3% S.E.M. when spiked at the 10 and 1 μg/ml levels, respectively. This indicates that TCAR does not partition to a significant extent from water into chloroform, which was confirmed by extracting an aqueous solution containing TCAR (100 μg/ml) with an equal volume (25 ml) of chloroform. The chloroform layer was evaporated to dryness and the residue dissolved in mobile phase (200 μl). No TCAR was detected (less than 40 ng/ml) in the residue by HPLC analysis.

Initial studies indicated that the processed plasma was incompatible with the chromatographic system, because complete blockage of the column resulted after injection of a single sample. This problem was traced to the

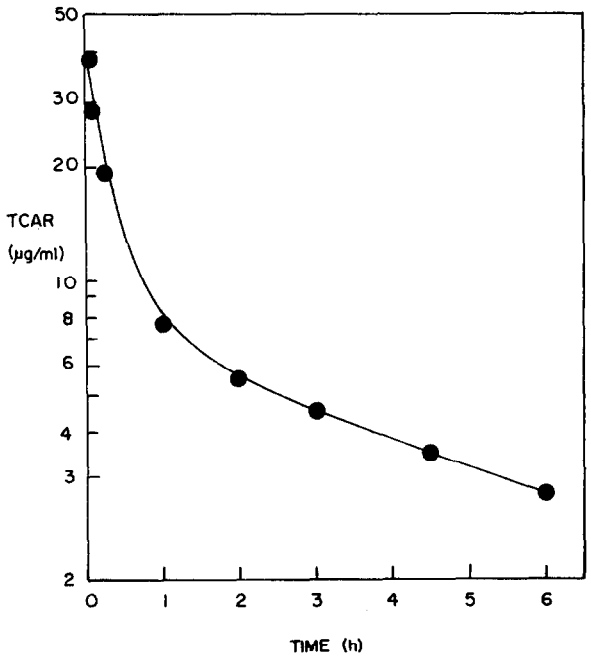

Fig. 7. Plasma concentrations of TCAR after intravenous administration (25 mg/kg) in the dog.

on-column precipitation of hexadecyltrimethylammonium perchlorate, and was circumvented by adjusting the HTAB concentration of the plasma extract to 1 m*M* and removing the resulting precipitate prior to injection into the chromatograph.

Application

The methodology was found to be suitable for the analysis of TCAR in both human and canine plasma and its applicability was demonstrated by an abbreviated pharmacokinetic study in the dog. The plasma levels of TCAR were followed for 6 h after its administration (25 mg/kg, intravenously) to a female Beagle dog, and the results are shown in Fig. 7. Over this time period, the drug exhibited biexponential decay with half-lives of 11 min (α-phase) and 4.0 h (β-phase). The extrapolated plasma concentration, at time zero, was 44.7 μg/ml which corresponds to a volume of distribution of 5.6 l.

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REFERENCES

1 J.M. Fuertes, T. Garcia-Lopez and M. Stud, J. Org. Chem., 41 (1976) 4074.
2 P.C. Srivastava, M.V. Pickering, L.B. Allen, D.G. Streeter, M.T. Campbell, J.T. Wilkowski, R.D. Sidwell and R.K. Robins, J. Med. Chem., 20 (1977) 256.
3 R.K. Robins, P.C. Srivastava, V.L. Narayarian, J.P. Plowman and K.D. Paull, J. Med. Chem., 25 (1982) 107.
4 H.N. Jayaram, R.L. Dion, R.I. Glazer, D.G. Johns, R.K. Robins, P.C. Srivastava and D.A. Cooney, Biochem. Pharmacol., 31 (1982) 2371.
5 J. Blanchard, J. Chromatogr., 226 (1981) 455.
6 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57.
7 G.J. Manius and R.J. Tscherne, Amer. Lab., (1981) 138.
8 C.M. Riley, L.A. Sternson and A.J. Repta, J. Chromatogr., 217 (1981) 405.
9 C.M. Riley, L.A. Sternson and A.J. Repta, J. Chromatogr., 219 (1981) 235.
10 C.M. Riley, L.A. Sternson, A.J. Repta and R.W. Siegler, J. Chromatogr., 229 (1982) 373.
11 J.J. Stranahan and S.N. Deming, Anal. Chem., 54 (1982) 2251.