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COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SERUM PROFILES OF HUMANS AND DOGS

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SUMMARY

The sera of 30 healthy male beagles were analyzed by reversed-phase high-performance liquid chromatography. The profiles were compared with those obtained from the sera of 30 healthy human donors. The chromatograms of each group were very reproducible; however, there were characteristic differences between the two groups. The compounds observed in both the human and canine profiles were identified as creatinine, uric acid, tyrosine, hypoxanthine, xanthine, kynurenine, inosine and tryptophan. Compounds present only in the canine profiles were identified as cytindine, riboflavin and 5-methylcytidine. Compounds present only in the human profiles include uridine, guanosine, hippuric acid and the dietary dependent compounds theobromine and caffeine. The compounds present in both human and canine sera were quantitated and compared statistically. The amounts of these compounds were very similar, except for uric acid.

INTRODUCTION

Biochemical assays of biological samples are useful in characterizing the abnormal processes of physiological disorders. In recent years, alterations in the concentrations of certain nucleosides and bases have been observed in the urine of patients suffering neoplastic diseases [1—5].

Although nucleosides and bases in urine have been studied extensively, their presence in serum has not, owing to the lack of adequately sensitive analytical methods. The development of microparticulate, reversed-phase packings for high-performance liquid chromatography (HPLC) has permitted the accurate, rapid and senstive determination of nucleosides and bases and other low-molecular-weight compounds present in serum at picomole levels [6—11].

Recent efforts in our laboratory have been directed at determining the normal range of values for the low-molecular-weight, UV-absorbing constituents of human sera, in order to evaluate changes that may occur as a result of disease processes [10—12]. We have also investigated the normal range

of low-molecular-weight, UV-absorbing compounds in the sera of male beagle dogs because beagles are used as models in studies of disease processes. Therefore, a comparative study of the constituents in human and the beagle sera was made.

MATERIALS AND METHODS

Instrumentation

A Waters Assoc. (Milford, Mass., U.S.A.) liquid chromatograph equipped with two Model 6000 A solvent delivery systems, a Model 660 solvent programmer, a Model U6K injector and a Model 440 dual-wavelength detector was used in all determinations. An on-line SF 770 Spectroflow Monitor and an FS 970 L.C. Fluorometer (Schoeffel Instruments Div., Kratos Inc., Westwood, N.J., U.S.A.) were also used. A two-channel Omniscribe recorder (Houston Instruments, Austin, Texas, U.S.A.) was used to monitor the 254- and 280-nm responses. Retention times and peak areas were obtained with an HP 3380-A electronic integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Materials

All standard reference compounds and high-purity enzymes were purchased from Sigma (St. Louis, Mo., U.S.A.). Solutions of the reference compounds and enzymes were prepared in doubly distilled, de-ionized water and buffered with reagent-grade potassium dihydrogen orthophosphate (Mallinckrodt, St. Louis, Mo., U.S.A.) at pH 7.0. Methanol was purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.).

Chromatographic conditions

Separations were obtained on microparticulate, chemically bonded, reversed-phase columns (300 × 3.9 mm I.D., μBondapak; Waters Assoc.) protected by a pre-column (50 × 4.6 mm I.D.) dry-packed with 25-μm, pellicular, reversed-phase material (Whatman, Clifton, N.J., U.S.A.). A 35-min linear gradient from 100% of the initial eluent (0.02 *F* potassium dihydrogen orthophosphate, pH 5.6) to 40% of the final eluent (60% methanol—water) was used to obtain rapid separations of the serum constituents. The flow-rate was 1.5 ml/min and the temperature was ambient.

Sample preparation

To obtain human serum samples, freshly drawn blood was collected from donors of similar age in Vacutainer tubes without anticoagulant. The donors had no known diseases and were not taking any medication. The blood was allowed to clot spontaneously for 10—15 min at ambient temperature. Then the tubes were centrifuged at 575 relative centrifugal force (RCF) for 10 min and the supernatant was allowed to sit over the clot for 3—4 h [11]. The supernatant was collected and filtered through Amicon membrane cones (Amicon, Lexington, Mass., U.S.A.) to remove protein and other materials with molecular weights greater than 25,000. The protein-free filtrates were transferred into polyethylene vials and stored at —20°.

Canine serum samples were obtained from a closed-colony group of male

beagle dogs of similar age, weight and size maintained at the Borriston Research Labs. (Temple Hills, Md., U.S.A.). Blood was drawn, allowed to clot and centrifuged in the manner described above. The supernatant was collected and transported frozen. Upon receipt, the samples were allowed to sit for 3 h at ambient temperature, then filtered through the membrane cones.

Each serum sample was analyzed in triplicate.

Peak identifications

Tentative identifications of the low-molecular-weight, UV-absorbing compounds present in the serum were based on comparisons of retention times and absorbance ratios ($area_{280}/area_{254}$) with those of reference compounds. Each standard reference compound which had a retention time and absorbance ratio similar to those of a peak in a serum chromatogram was co-chromatographed with the serum sample to determine if the standard co-eluted and if the absorbance ratios were constant.

To further support identifications, enzymatic peak shifts and UV and fluorescence spectra were obtained for the peaks in the chromatograms [10, 11].

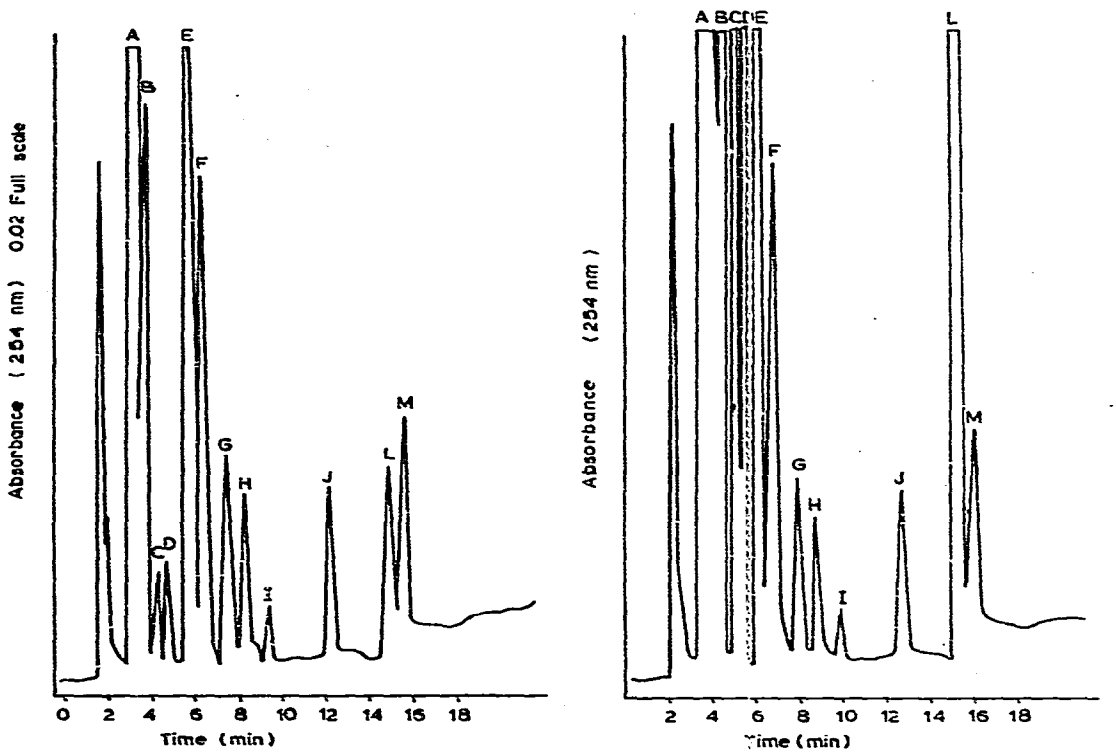

Fig. 1. Chromatogram of beagle serum constituents. Injection volume: 40 µl. Chromatographic conditions listed in text.

Fig. 2. Serum sample illustrated in Fig. 1 co-chromatographed with the standards creatinine (A), uric acid (B), tyrosine (C), cytidine (D) and tryptophan (L).

For the enzymatic peak shift, a 10-μl aliquot of enzyme solution specific to the peak of interest was added to a 50-μl aliquot of serum, the reaction mixture chromatographed and the profile compared with that of the untreated serum sample.

The UV spectra of the peaks of the serum profiles were obtained by a stopped-flow scanning technique [8]. Background interference was stored in the detector memory module, where it is automatically subtracted to give an accurate spectrum of the serum peaks as they eluted from the column.

Fluorescence was used selectively to detect and identify uric acid, tyrosine, kynurenine and tryptophan in the serum profiles [7, 11]. Further, as few naturally occurring serum constituents fluoresce, it is possible to characterize these biologically important compounds without interferences.

RESULTS

The identification techniques used in determining the compounds present in the human and canine HPLC profiles are illustrated with a beagle serum sample (Fig. 1). The peaks of the profile were first identified by co-chromatographing the serum sample with standard reference compounds that had similar retention times and absorbance ratios to those of peaks in the serum profile. Increases in peak areas caused by the co-eluting compounds and constant absorbance ratios (Fig. 2) indicate that peaks with the retention times of creatinine, uric acid, tyrosine, cytidine and tryptophan are present in the sample. Peaks were further characterized by obtaining UV spectra of each peak

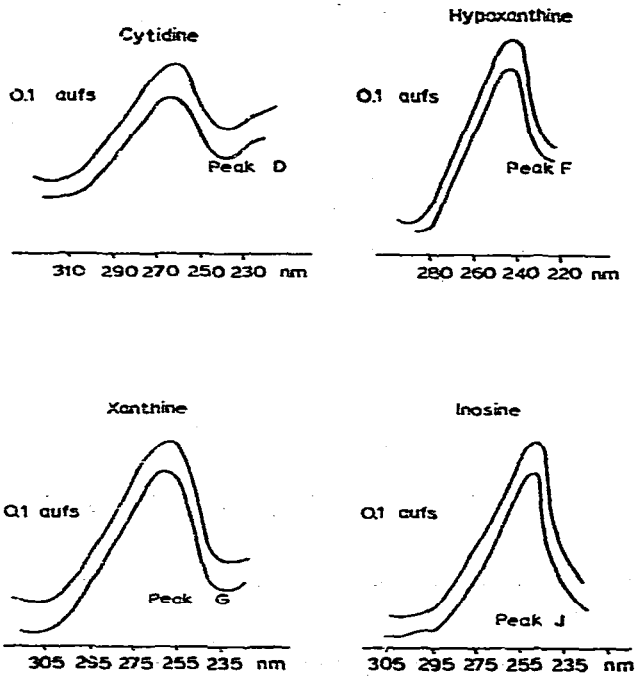

Fig. 3. UV spectra obtained with the stopped-flow technique from canine serum and reference compounds.

in the serum profile. The spectra of the serum peaks were then compared with those of standard reference compounds (Fig. 3). Chromatograms using fluorescence detection were obtained with an excitation wavelength of 285 nm and an emission cut-off filter of 320 nm, which indicated that the three peaks in Fig. 4 were tyrosine, kynurenine and tryptophan. Finally, commercially available enzymes specific to the compound of interest were used to confirm peak identity by reacting an aliquot of enzyme solution with the serum sample. For examlpe, an aliquot of serum that was incubated with a xanthine oxidase solution for 25 min was chromatographed. The chromatogram was then compared with the profile of the untreated serum sample (Fig. 5). The disappearance of two peaks in the profile supports the identification of peaks F and G as hypoxanthine and xanthine. Therefore, using a combination of data obtained with the various techniques, the peaks of the canine profiles were identified as (A) creatinine, (B) uric acid, (C) tyrosine, (D) cytidine, (F) hypoxanthine, (G) xanthine, (J) inosine and (L) tryptophan. Peaks E, H and I were tentatively identified as riboflavin, kynurenine and 5-methylcytidine, respectively. Peak M remained unidentified.

The same identification procedures were used to determine the peaks of the human serum profiles [11]. A comparison of the human and canine profiles is

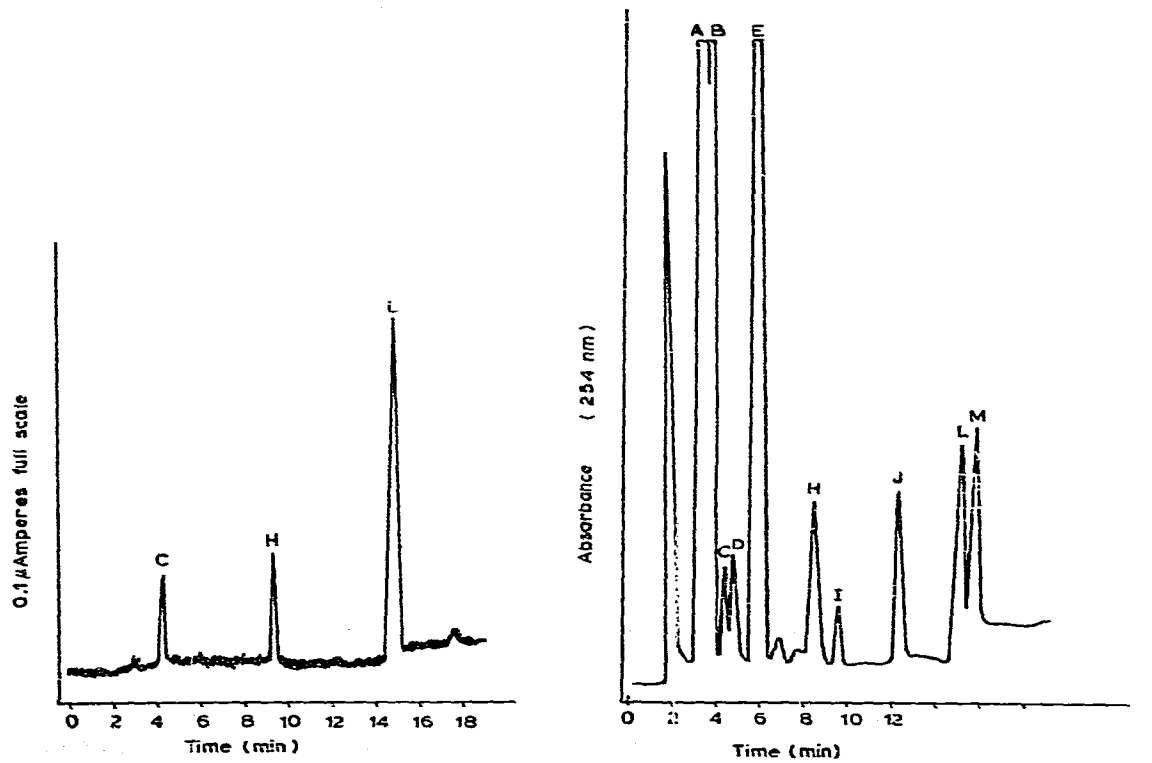

Fig. 4. Canine serum detected with fluorescence. Excitation wavelength: 285 nm. Emission cut-off wavelength: 320 nm. Peaks: C = tyrosine, H = kynurenine, L = tryptophan.

Fig. 5. Canine serum sample reacted with xanthine oxidase. See text for conditions. Note the absence of peaks F (hypoxanthine) and G (xanthine), the increase in peak B (uric acid) and no change in any of the other peaks.

shown in Fig. 6. Creatinine, uric acid, tyrosine, hypoxanthine, xanthine, kynurenine, inosine and tryptophan were found in the sera of both humans and beagle dogs. Uridine, guanosine, hippuric acid, theobromine and caffeine were found only in the human sera, while compounds with the retention times of cytidine, 5-methylcytidine and riboflavin were found only in canine sera.

The compounds present in the serum profiles were quantitated by an external standard method. Response factors for each known peak were determined by chromatographing a known amount of the standard reference compounds. The response factors were linear over the range of concentrations found in serum. An average value of triplicate analyses of the serum

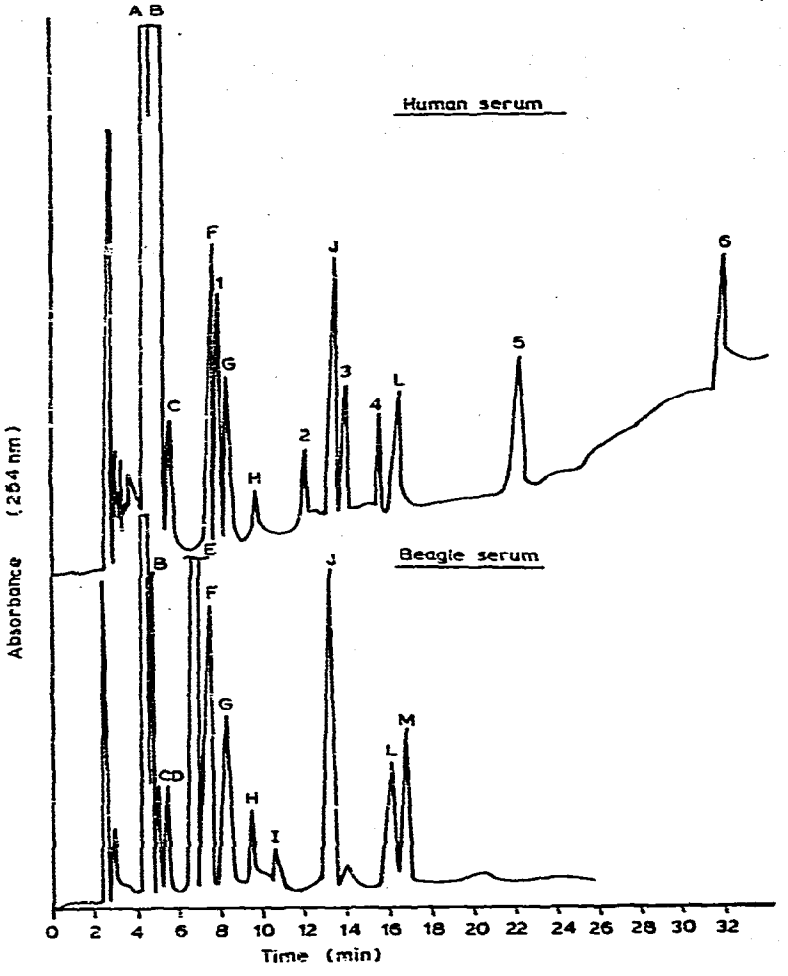

Fig. 6. Comparison of the low-molecular-weight, UV-absorbing serum constituents of humans and beagle dogs. Injection volume: 40 μl. Components of human serum: creatinine (A), uric acid (B), tyrosine (C), hypoxanthine (F), uridine (1), xanthine (G), kynurenine (H), unknown (2), inosine (J), guanosine (3), hippuric acid (4), tryptophan (L), theobromine (5) and caffeine (6). Components of canine serum that differ from those of human serum: cytidine (D), riboflavin (E), 5-methylcytidine (I) and unknown (M). The numbered peaks are those which do appear in the chromatogram of the human serum but not in that of the beagle serum.

TABLE I

CONCENTRATIONS OF LOW-MOLECULAR-WEIGHT UV-ABSORBING COMPOUNDS IN BEAGLE AND HUMAN SERA

Compound	Concentration ± standard deviation (μmole/l)	
	Beagle serum	Human serum
Creatinine (Cre)	89.1 ± 30.6	83.1 ± 11.3
Uric acid (UA)	6.96 ± 10.6	295 ± 39.0
Tyrosine (Tyr)	43.2 ± 12.5	62.2 ± 11.3
Cytidine (Cyd)	1.90 ± 0.82	—
Riboflavin (Rbf)	209 ± 59.0	—
Hypoxanthine (Hyp)	8.95 ± 3.33	7.16 ± 2.81
Uridine (Urd)	—	3.17 ± 1.11
Xanthine (Xan)	8.72 ± 2.25	2.62 ± 1.04
Kynurenine (Kyn)	116 ± 40	103 ± 48
5-Methylcytidine (5mCyd)	2.66 ± 0.84	—
Inosine (Ino)	4.85 ± 1.40	5.62 ± 2.87
Guanosine (Guo)	—	0.881 ± 0.515
Hippuric acid (HA)	—	0.613 ± 0.477
Tryptophan (Trp)	55.4 ± 13.2	13.7 ± 3.57
Theobromine (Thb)	—	0 to 6.35 (dietary)
Caffeine (Caf)	—	0 to 15.6 (dietary)

components was used in the quantitations. Table I lists the average values and normal range of values for the low-molecular-weight, UV-absorbing compounds present in the sera of beagle dogs and humans.

The data from the human serum profiles were compared statistically with the data obtained from the canine serum profiles using a two-group comparison test based on Student's *t*-distribution. The levels of the compounds present in both the human and canine serum were not significantly different (95% confidence limit), except for uric acid, the amount of which was significantly greater in the human sera than in the canine sera.

DISCUSSION

Recently, reports on the identification and quantitation of some low-molecular-weight, UV-absorbing constituents of human sera by HPLC have appeared [10, 11]. Although there are a number of reports on the high-molecular-weight constituents of canine sera [13–16], little has been reported on the low-molecular-weight constituents, such as nucleosides and bases.

However, in the plasma of mongrel dogs, Tseng et al. [17] found that the levels of two compounds, cytidine and uric acid, were 1.89 and 0.367 μM respectively. In addition, uridine was found to be converted rapidly into uracil in the plasma of these dogs by enzymes not present in human plasma. On the other hand, cytidine was converted into uridine in human plasma by enzymes not present in the dogs. Our results support these findings because cytidine was found in the canine sera whereas uridine was not.

In addition, 5-methylcytidine, which has been found in some mammalian

cells [18, 19], appears to be a normally occurring serum constituent in beagle serum although not in human serum.

In man, the end product of purine metabolism is uric acid; however, in many animals and birds the end product is allantoin. The low levels of uric acid in the canine sera support the fact that there is a higher activity of the enzyme uricase in beagles than in humans.

As there are similar concentrations of most nucleosides and bases in the sera of humans and beagles, it appears that the major purine metabolic pathways are similar, even though there are significant differences in the pyrimidine pathways. Therefore, beagles can be used as models of the human system in certain experiments related to purine metabolism, although it remains to be seen whether there are other animal species more closely related to humans in their serum constituents.

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