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EFFICIENT CHROMATOGRAPHIC FRACTIONATION OF STEROIDS IN HUMAN SERUM THROUGH REGULATION OF LIQUID—LIQUID DISTRIBUTION RATIOS

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SUMMARY

To improve the clean-up process in the analysis of biological fluid constituents, an efficient liquid—liquid distribution system was developed. Closed-bed columns containing fine diatomaceous earth granules were prepared by slurry packing for the fractionation of steroid hormones in human serum before quantitative assay by liquid chromatography or radioimmunoassay. Four columns were connected to construct the aqueous liquid—liquid chromatography—fractionation system. The first was coated with neutral water for distribution of serum, the second was weakly alkaline with sodium hydrogen carbonate for extrusion of strong acidic components, and the third was strongly alkaline with sodium hydroxide to capture oestrogens. The final column was acidic with sulphuric acid to remove basic components. Optimization of the stepwise gradient solvents was achieved on the basis of the results of a linear relationship between the logarithms of the capacity ratios and solvent composition determined from an analytical run. Neutral steroid hormones added to serum were eluted from the column system by a stepwise gradient elution technique to obtain first very non-polar materials, then progesterone and testosterone, and finally to extract the corticosteroids. Phenolic oestrogens were recovered from the strong alkaline column with a mobile phase solvent after the pH of the stationary phase had been adjusted with a phase transfer neutralizer. The fractional constituents were purified and enriched. This procedure was used to determine Solu-medrol, an acidic corticosteroid drug, in human serum.

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INTRODUCTION

To improve the analytical efficiency and sensitivity of samples consisting of complex matrices such as biological fluids, a clean-up procedure including preliminary fractionation is often ideal. Solvent extraction has been commonly used to fractionate target molecules in a complex mixture. We recently developed a highly efficient aqueous liquid—liquid distribution procedure using closed-bed columns containing fine silica gel or diatomaceous earth granules coated with an aqueous stationary phase [1—3]. The selectivity of this method is based on specific distribution ratios of solutes in organic mobile and aqueous stationary phases; in addition, two phases prepared on the surface of the packing materials participate in the distribution of a high theoretical plate number. A switching technique from the precolumn for aqueous liquid—liquid distribution to the high-performance liquid chromatographic (HPLC) analytical column was applied to the analysis of biological fluids [4, 5]. An on-line extraction— evaporation—injection technique for improving the clean-up of serum for analysis of corticosteroids and certain other drugs has been described in our earlier papers [5, 6].

For greater efficiency of the clean-up process, we present in this paper an improved fractionation procedure employing highly selective aqueous liquid—liquid distribution by which the partition coefficients of the solutes are regulated. If a series of columns are coated with acidic and basic stationary liquids, basic and acidic solutes can be eliminated, and consequently unretained neutral components emerge from the column system so that a pure fraction is finally obtained. The basic and acidic components in a mixture can be recovered by application of a neutralization reagent, adjusting the pH of the stationary phases and solvent extraction. The distribution ratios of the solutes depend on the nature of the organic phase systems; for example, hydrophobic substances are easily distributed in a more lipophilic organic layer and are eluted more quickly than hydrophilic ones. The characteristics of the organic phase can be regulated by mixing two solvents of different polarity. A linear correlation between the logarithms of solvent composition and distribution ratio has been found recently for binary solvent systems using various solute compounds in liquid—liquid chromatography [3, 7]. It is possible to control the distribution ratio of a solute by altering the binary solvent composition in accordance with solute polarity and optimizing the fractionation process in a systematic way.

To carry out the process described above, a trial run was made using steroids in biological fluids as model compounds. Steroids added to human serum were fractionated into oestrogens, corticoids and other neutral steroids, following a time-scheduled sequential operation. An acidic steroid drug, Solu-medrol (6-methylprednisolone 21-hemisuccinate), in human serum was fractionated quantitatively to test the application of this process to clinical assay.

EXPERIMENTAL

Apparatus

The system used in the experiments is illustrated in Fig. 1. Glass tubes 1, 2

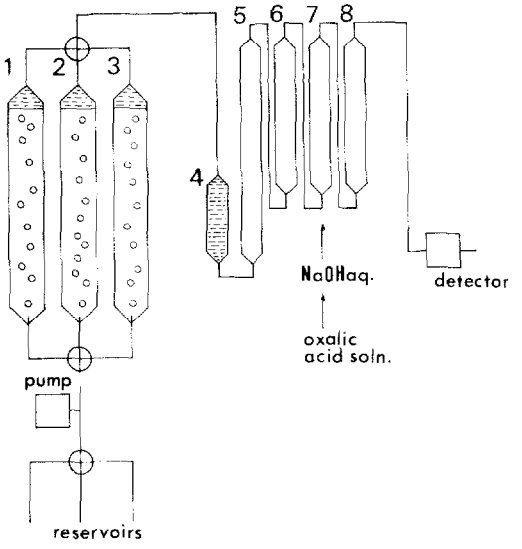


Fig. 1. Schematic diagram of the system. 1—3: Glass tubes containing water; 4: glass tube for injection; 5—8: diatomaceous earth columns coated with water, 5% sodium bicarbonate, 3% sodium hydroxide and 1% sulphuric acid solutions, respectively.

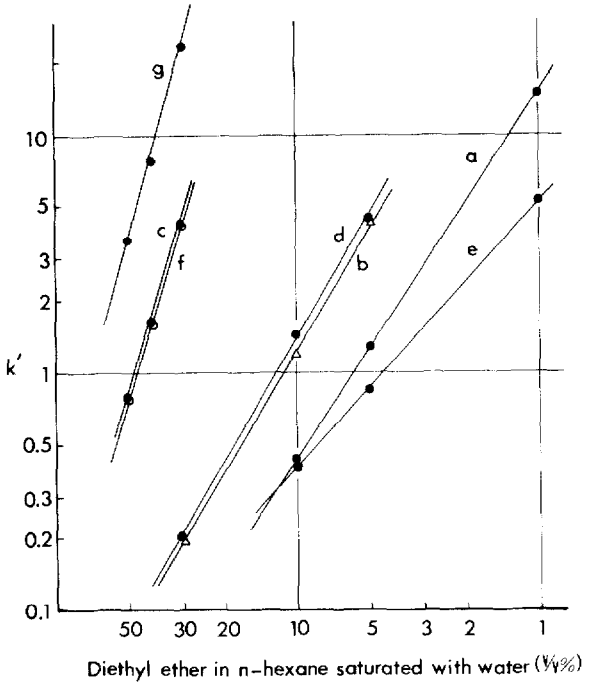


Fig. 2. Correlation between the logarithm of capacity ratio and that of aqueous binary solvent composition in steroid samples. Packing material: diatomaceous earth (AquEx-10, DSD). Samples: a = oestrone, b = oestradiol, c = oestriol, d = testosterone, e = progesterone, f = corticosterone, g = hydrocortisone.

and 3 (30 cm × 2.5 cm I.D.), each containing about 100 ml of water, were used to presaturate the carrier solvent with water as described previously [5, 6]. Glass tube 4 and column 5 were used for the injection and retention measurements of steroid hormones (procedure a). An additional three columns, 6, 7 and 8, were used to fractionate the serum steroids (procedure b). These columns were packed with diatomaceous earth granules using a slurry packing procedure [2, 6]. Tube 4 and columns 5—8 were connected in cascade with PTFE tubes 30 cm × 1 mm I.D. The inlet of tube 4 was connected to glass tubes 1, 2 and 3 and a pump (Twincle, Jasco, Tokyo) via PTFE valves, and the outlet of column 5 (for procedure a) or column 8 (for procedure b) was connected to a UV detector (Uvidec III, Jasco). Selection of tube 1, 2, or 3 in accordance with the carrier solvent was made using a PTFE valve inserted between the pump and tubes 1, 2 and 3. The glass tubes and column tubes (CIG tubes) were obtained from Kusano (Tokyo, Japan). The other mechanical components were the same as described in our earlier papers [5, 6].

Reagents and solvents

The steroid hormones and reagent grade chemicals were supplied from Wako (Osaka, Japan). An authentic sample of Solu-medrol was obtained from Japan Upjohn. Diatomaceous earth for the column extraction, particle size 10 μm, AquEx-10 was obtained from DSD (Tokyo, Japan).

Procedure a: determination of the linear relationship between the logarithm of the capacity ratio and that of the eluent composition

Column 5, 17 cm × 4 mm I.D., containing diatomaceous earth, was washed

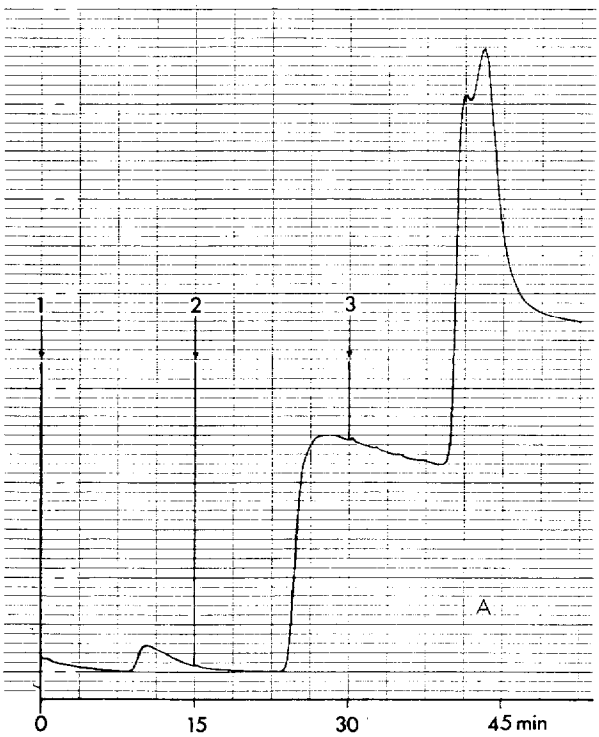
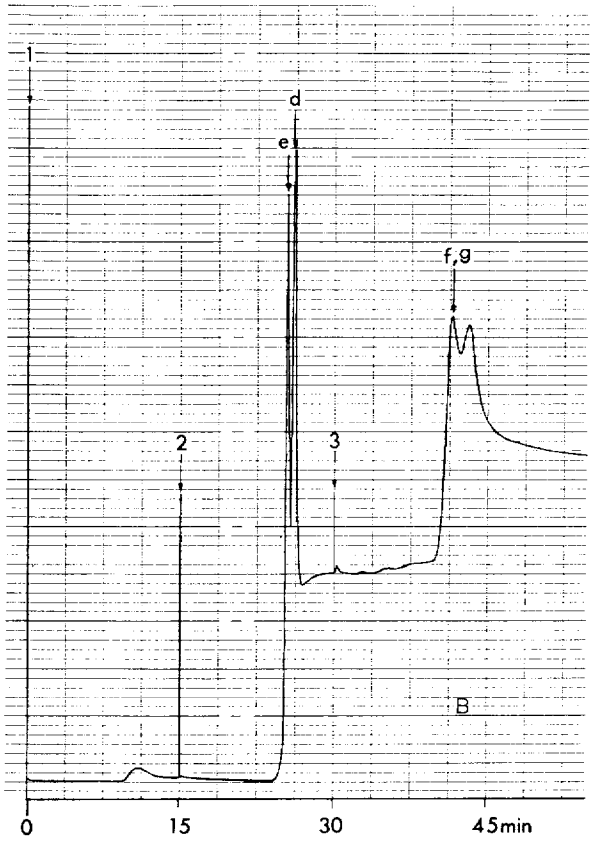


Fig. 3.



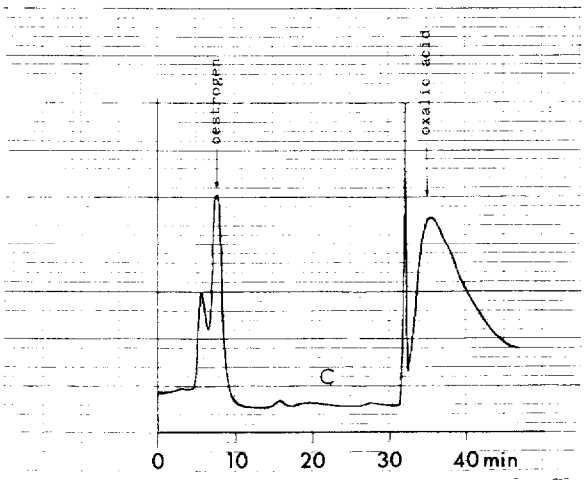


Fig. 3. Chromatograms of steroid fraction obtained by the stepwise elution of serum. (A) Steroid fractions in normal serum (0.6 ml). (B) Neutral steroids added to the serum (0.3 ml). (C) Oestrogens added to the serum (0.3 ml). 1 = Elution by 0.1% diethyl ether in *n*-hexane; 2 = elution by 30% diethyl ether in *n*-hexane; 3 = elution by diethyl ether. Samples d—g as in Fig. 2.

with 6 ml each of methanol and water. The column was washed again with water-saturated diethyl ether applied at a flow-rate of 9.9 ml/min for 5 min to remove any excess aqueous phase. The column contained about 0.1 ml of aqueous phase. The glass tube injector 4 was a CIG tube, 7 cm × 4 mm I.D. The UV detector was set at 240 nm for testosterone, progesterone and corticosteroids, and at 280 nm for oestrogens. The analytical column 5 thus constructed showed theoretical plate numbers ranging from 700 to 1200 for each steroid hormone and a dead volume of about 2.0 ml.

Before the analytical run, an equilibration was made between the aqueous stationary and mobile phases, consisting of a mixture of water and diethyl ether—*n*-hexane made by shaking these components together. The solvent-free injection of the sample was carried out as follows: 10 μl of a 2-propanol solution containing 2 μg of steroid hormones were smeared on a glass rod, 6.8 cm × 3.7 mm O.D. After the solvent had dried, the rod was inserted into the injector and the mobile phase solvent was slowly introduced at a flow-rate of 0.1 ml/min. When the solvent reached the analytical column, the flow-rate was adjusted to 1 ml/min and the eluent was monitored by the UV detector. The mobile phase solvents were *n*-hexane and mixtures of diethyl ether and *n*-hexane with diethyl ether concentrations of 1, 5, 10, 30, 40 and 50 vol.%. The experimental values of the capacity ratios were obtained by the formula $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention time and dead time, respectively. The correlation between the logarithm of capacity ratio and that of solvent composition is shown in Fig. 2.

Procedure b: fractionation of the serum steroids

A blood sample (5 ml) was centrifuged for 5 min at 1000 *g*. Tube 4 (7 cm × 4 mm I.D.) was used to inject up to 0.8 ml of serum supernatant. Columns containing diatomaceous earth were 30 cm × 4 mm I.D. (column 5) and 20 cm × 4 mm I.D. (columns 6—8).

Fractional columns 5, 6, 7, and 8 were conditioned with distilled water, 5% sodium bicarbonate solution, distilled water, and 1% sulphuric acid solution, respectively. A 100-μl volume of 3% sodium hydroxide solution was injected into column 7 at its bottom followed by an injection of 0.6 ml of serum into column 4. Columns 5, 6, 7 and 8 thus prepared were neutral, weakly alkaline, strongly alkaline and acidic, respectively. A mixture of 0.1% diethyl ether—*n*-hexane was introduced into column 4 via tube 1 at a flow-rate of 0.8 ml/min. The serum in column 4 was transferred to column 5 where it remained in the aqueous stationary phase. The mobile phase was transferred through the column system and finally entered the detector set at 240 nm. After 15 min the mobile phase solvent was changed to a mixture of 30% diethyl ether—*n*-hexane, and then to diethyl ether after 30 min. The results are shown in Fig. 3A.

This same procedure was carried out with 0.3 ml of serum added along with 2 μg each of progesterone, testosterone, corticosterone, and hydrocortisone, and 4 μg each of oestrone, oestradiol, and oestriol. Eluents within 24—27 min and 40—44 min were collected. The first fraction contained progesterone and testosterone, and the second corticosterone and hydrocortisone. The chromatogram obtained is shown in Fig. 3B.

In the next stage, in order to recover the oestrogens remaining in the

strongly alkaline stationary phase at the bottom of column 7, the column system was rearranged as follows. Columns 4, 5, and 6 were disconnected and column 7 was connected directly to tube 3. After 0.2 ml of saturated oxalic acid solution had been injected into column 7, water-saturated diethyl ether was introduced at a flow-rate of 0.8 ml/min. The eluent from column 8 was monitored by the detector set at 280 nm and within 5—10 min collected so as to obtain the oestrogen fraction. The chromatogram obtained is shown in Fig. 3C.

For extraction of Solu-medrol in the serum, a process similar to that used for the oestrogens was carried out on 0.3 ml of serum supplemented with 10 ng of the steroid drug and 10 μl of 50% hydrochloric acid solution to bring the pH to about 4—5. The Solu-medrol trapped in the weak alkaline stationary phase in column 6 was recovered by injection of a saturated oxalic acid solution and extraction with water-saturated diethyl ether.

Extracts containing the above steroids were applied to the on-line evaporation—injection system described in earlier papers [5, 6]. The steroids were identified by their retention data, using silica gel columns and a normal-phase solvent system containing *n*-hexane—2-propanol for steroid hormones and *n*-hexane—2-propanol—water—acetic acid for Solu-medrol. To check recovery, peak height measurements were made.

RESULTS AND DISCUSSION

From the linearity between the logarithm of capacity ratio and that of solvent composition illustrated in Fig. 2, optimization for selective fractionation was carried out. The retention behaviour in the figure indicates that progesterone and testosterone may possibly be retained to a fair extent and corticosteroids, such as corticosterone and hydrocortisone, strongly retained in the column as a result of using the diethyl ether—*n*-hexane mixture. Thus, two classes of neutral steroids can be accurately fractionated by stepwise elution. The retention behaviour of oestrogens such as oestrone, oestradiol, and oestriol differed so much that their extraction as a single fraction with the diethyl ether—*n*-hexane mixture was not possible. To solve this problem, a column containing an alkaline stationary phase was used to trap the phenolic oestrogens.

After washing out the non-polar materials from the human sera with 0.1% diethyl ether—*n*-hexane (Fig. 3A), progesterone and testosterone were recovered from the columns by a 30% diethyl ether—*n*-hexane mixture. The columns used in this study, which were packed with fine diatomaceous earth granules, have a higher number of theoretical plates in comparison with commercially available cartridge-type columns. As a result, the hormones could be fractionated with only 2—4 ml of the mobile phase solvent, as shown in Fig. 3B. Normal serum components extractable by this solvent were present in rather small amounts so that the fraction obtained was enriched with these steroid hormones. Finally, the mobile phase solvent was changed to diethyl ether and corticosteroids such as corticosterone and hydrocortisone were fractionated.

During the course of this process, the oestrogens continued to remain in the strongly alkaline column 7 as a result of salt formation. Following comple-

tion of the neutral hormone fractionation, the stationary phase in column 7 was neutralized by a phase transfer neutralizer such as an oxalic acid solution to recover the oestrogens in the mobile phase. The results in Fig. 3C indicate a recovery with only 4 ml of the mobile phase solvent. The observed time delay in the oestrogen peak from the hold-up time indicates the time necessary for neutralization of the alkaline stationary phase with oxalic acid. An excess amount of oxalic acid was removed 22 min following completion of the oestrogen elution. Thus, it was quite easy to recover the oestrogens without any contamination from the reagent added for neutralization.

To extend the scope of this method and apply it to clinical assay, an acidic steroid drug, Solu-medrol, was selected for testing. This drug was added to the serum and extracted quantitatively after being trapped in the weak alkaline column and adjusting the pH of the stationary phase.

Steroid calibration curves were made from peak height measurements using

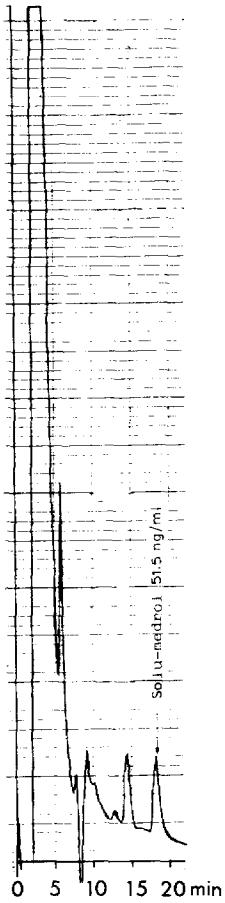


Fig. 4. Chromatogram of serum Solu-medrol obtained using the on-line system for extraction—evaporation—injection into HPLC column 7 using 0.1 ml of serum from a systemic lupus erythematosus patient. The eluent was *n*-hexane—2-propanol—water—acetic acid (72.5:25:2:0.5) for the silica gel column. The flow-rate was 1 ml/min. A detector range of 0.005 a.u.f.s. and recorder range of 10 mV were used.

sample quantities between 10 and 100 ng/ml of serum. The peak heights were reproducible with the coefficient of variation being less than 4%, using authentic samples injected into the analytical column without extraction. These experiments were carried out using the extraction—evaporation—injection system described in our earlier papers [5, 6]. The above method for liquid chromatographic analysis of serum Solu-medrol has found application in clinical assay. Fig. 4 shows a chromatogram obtained from the serum of a patient with a collagen disease administered Solu-medrol. The results of this application will be published in detail.

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