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RAPID, DIRECT DETERMINATION OF TRACE AMOUNTS OF SALICYLIC ACID IN DEPROTEINIZED SERUM BY MEANS OF HIGH-PRESSURE LIQUID—LIQUID CHROMATOGRAPHY

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SUMMARY

A simple method for the quantitative analysis of salicylic acid in blood serum is described. A liquid—liquid chromatographic system, consisting of a long-chain aliphatic amine as the stationary phase and dilute aqueous perchloric acid as the mobile phase, enables the direct injection of deproteinized serum into the system. No change in the chromatographic properties of the system was noticed after 2000 injections of deproteinized serum.

Quantitative analysis is possible using peak area or peak height measurements. The method has a high precision: relative standard deviations of 0.4% and 5% are found for samples containing 10 μg and 10 ng injected salicylic acid respectively. The detection limit is found to be about 1 ng slicylic acid, corresponding to 40 ppb* salicylic acid in serum.

Simultaneously administered drugs such as indomethacin, acetylsalicylic acid, caffeine and phenacetin, and metabolites of salicylic acid do not interfere with the analysis. The time course of the concentration of salicylic acid in serum is demonstrated after oral administration of 1 g sodium-salicylate. The phase system was also found to be suitable for the analysis of salicylic acid in urine.

INTRODUCTION

The favourable effect of aspirin in general and of sodium salicylate in particular as a medicine for the treatment of rheumatism is commonly accepted [1]. As a result of the very short half-life of aspirin (ASA) in man [2] and the rapid excretion of possibly formed metabolites [3,4], salicylic acid (SA) has to be considered as the active compound.

A rapid determination of SA in serum is essential for the adjustment of a medication schedule. This adjustment is necessary in order to find a suitable compromise between the therapeutic action and unfavourable side effects [5].

*Throughout this article, the American billion (10^9) is meant.

Usually, laborious extraction procedures are applied prior to the colorimetric or fluorimetric determination of SA [6,7]. Recently, gas—liquid chromatographic (GLC) methods for the simultaneous determination of ASA and SA in biological fluids have been described [2,8]. However, time-consuming extractions and derivatization are necessary in these methods. High-performance liquid chromatography (HPLC) has proved to be an excellent method for the determination of non-volatile acidic compounds in body fluids [9—11]. Until now, some papers have appeared dealing with the analysis of ASA, SA and other constituents in tablet preparations of these drugs by HPLC [12, 13].

In the present paper a rapid determination of SA in deproteinized serum using high-pressure liquid—liquid chromatography with UV detection is described.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of: reciprocating membrane pump (Orlita DMP 1515, Giessen, G.F.R.); flow-through manometer as damping device; stainless-steel 316 precolumn (350 mm × 10 mm I.D.); high-pressure sampling valve (Valco CV-6-UHPa) with a loop of 135 μl; thick-walled glass column (150 mm × 3 mm I.D., 12 mm O.D.) or stainless-steel 316 column (150 mm × 3 mm I.D., 6.4 mm O.D.); UV detector (variable wavelength, Zeiss PM 2 DLC, Zeiss, Oberkochen, G.F.R.); linear potentiometric recorder (Goertz, Servogor 542) combined with an integrator (Spectra-Physics Autolab System I).

In order to resist the acidic medium all connections were made of stainless-steel 316 capillary tubing and stainless-steel Swagelok couplings. The experiments were carried out at room temperature. The wavelength was adjusted to 235 nm.

Materials

In all experiments double-distilled water was used. Tri-*n*-octylamine (TOA) was from Fluka (Buchs, Switzerland). As solid support for the glass column diatomite (5—7 μm Kieselguhr, Merck, Darmstadt, G.R.F.) was used, and for the stainless-steel column low-surface-area silica (4—6 μm Spherosil XOC 005, Rhône-Poulenc, Neuilly-sur-Seine, France) was used. Materials of appropiate particle size range were prepared by means of an air classifier (Alpine MZR, Augsburg, G.F.R.). Spherosil XOB 015 (100—200 μm, Rhône-Poulenc) was used as solid support for the precolumn. All chemicals were commercially available and of analytical grade.

Procedures

Coating procedure. The solid supports were coated by a solvent evaporation technique. To 1 g solid support 0.04 g TOA dissolved in 10 ml dichloromethane was added. After stirring the slurry, the dichloromethane was removed by evaporation while stirring under a stream of dry nitrogen until the support was completely dry.

Packing procedures. The glass columns were dry-packed with coated diato-

mite. Small portions of about 10 mg coated support were put into the glass tube and compressed by hand with a teflon-tipped plunger.

The stainless-steel columns were packed by a high-pressure slurry technique using a packing apparatus as shown in Fig. 1. The column was connected to a metallic mixing vessel filled with a slurry of coated Spherosil XOC 005 in 0.05 *M* aqueous perchloric acid (2%, w/w) saturated with TOA. The slurry was pumped upward into the column at a liquid stream of 3 ml/min up to a pressure of 500 atm.

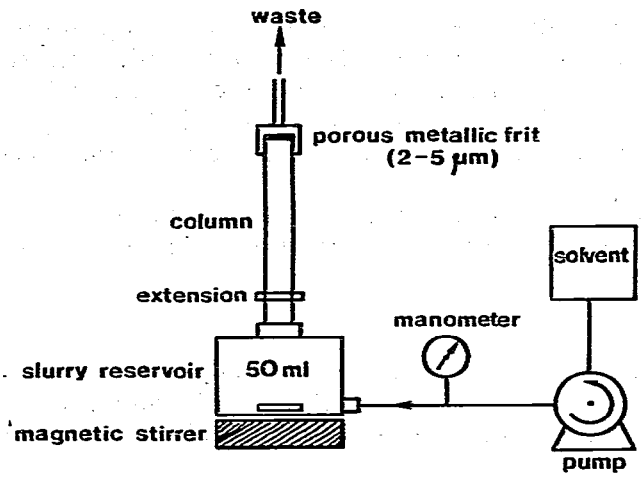

Fig. 1. Apparatus for slurry packing procedures in HPLC.

The precolumn was loosely dry-packed with Spherosil XOB 015 coated with TOA (10% w/w). Before use 250 ml of the eluent were pumped through in order to saturate the stationary phase with perchloric acid.

Mobile phase preparation. The mobile phase was prepared by diluting a weighed amount of perchloric acid (70%, w/w) with double-distilled water. The eluent was saturated with TOA and ultrasonicated to remove air.

Sample preparation. In all experiments centrifuged (10 min, 300 *g*) blood serum, free of exogenous compounds, was used. Batches of serum were stored in a deep-freeze.

To deproteinize 0.2 ml serum was mixed with 1 ml 0.3 *M* perchloric acid (pH 0.7) in a plastic centrifuge tube (10 ml). After 10 min the proteins were removed by centrifugation (10 min, 300 *g*). The supernatant was injected directly into the column using a sample loop of 135 µl volume. The loop was rinsed with water after each injection to prevent contamination and memory effects.

RESULTS AND DISCUSSION

Phase system

The determination of SA in serum was investigated using a liquid–liquid

system consisting of TOA as the stationary phase and dilute aqueous perchloric acid as the mobile phase [14,15].

The most simple method for the analysis of SA should be direct injection of diluted serum into the chromatographic system. In practice, however, this restricts the choice of the mobile phase as precipitation occurs at pH < 3, thus blocking the column.

Direct injection of 30-times diluted serum, using 0.05 *M* $HClO_4$ + 0.055 *M* Na_2HPO_4 (pH 5.5) as the mobile phase, seriously disturbed the chromatographic system as a result of the very large background. Repeated injections gave rise to baseline drifts and column blocking probably caused by precipitation of proteins at the top of the column.

The serum must therefore be deproteinized before injection. This prevents precipitation of proteins and significantly improves the background. This allows, moreover, the use of a more acidic eluent. This is favourable as the deproteinization can be performed with $HClO_4$ of such concentration that the supernatant has an identical pH and anion concentration as the eluent, while the dilution of the sample is kept minimal.

For this reason 0.25 *M* $HClO_4$ (pH 0.7) was chosen as the mobile phase. As shown in Fig. 2, with this phase system SA can be separated from residual serum compounds within six minutes.

Quantitative aspects of the method

The influence of serum constituents on the quantitative analysis of SA was investigated. A constant volume (135 µl) of solutions of SA in the eluent or in the supernatant of deproteinized serum was injected into the column and the

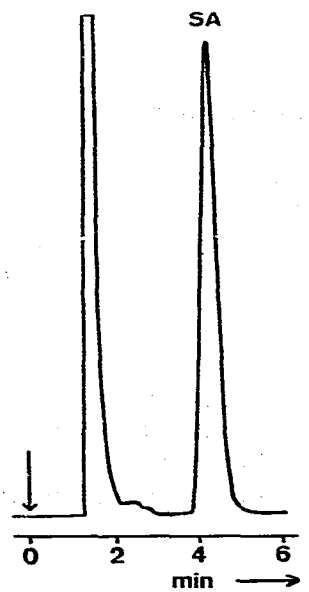

Fig. 2. Analysis of SA in deproteinized serum.
Phase system 4% (w/w) TOA on Kieselguhr—0.25 *M* $HClO_4$, pH 0.7. Glass column, 150 × 3 mm; injection volume 135 µl, Δp = 100 bar.

peak areas were measured. The relative standard deviation of the chromatographic procedure was found to be 0.4% (n=20) for injections of a standard solution of SA (4 μg/ml) in the eluent. No difference could be noticed between the linear regressions of peak area versus injected amount obtained with SA dissolved in the eluent or in deproteinized serum. Both linear regressions showed an equal slope over the range 10—20.000 ng SA with a correlation coefficient of 0.9999.

Relative standard deviations of 0.4% and 5% were found for samples containing 10 μg and 10 ng injected SA respectively (n=4).

To investigate the effect of deproteinization on the recovery of SA, known amounts of SA dissolved in water were added to serum before deproteinization. The recovery of SA varied between 90% in the μg range to 85% in the ng range. The recovery can be increased to 90—97% by washing the precipitated proteins two times with 1.2 ml eluent. In the determination of SA in serum after deproteinization there was a linear relationship between peak area and amount of SA injected over the range 10—20.000 ng, with a correlation coefficient of 0.9999. Since the same relative standard deviations were found as for SA dissolved in the eluent, the reliability of the method is not affected by the deproteinization.

Since the peak of SA in the phase system TOA—$HClO_4$ is very symmetrical, peak heights instead of peak areas can be used for quantitative analysis. The linear regression of peak height versus amount of SA injected measured within the range 20—10.000 ng showed a correlation coefficient of 0.9999, indicating a high degree of linearity.

The detection limit of SA, defined as 3 times the standard deviation of the noise, is found to be about 1 ng. This corresponds with 40 ppb SA in serum for the given injection volume of 135 μl, the largest volume that can be injected without loss of column efficiency.

Pharmacokinetic study

Simultaneously administered drugs such as caffeine, phenacetin and ASA do not disturb the analysis of SA since they are well resolved from SA.

Fig. 3 shows the analysis of serum from a human subject, analysed 45 min after oral administration of 200 mg ASA, phenacetin and caffeine.

SA and a little phenacetin were found while caffeine and possible residues of ASA, compounds eluted before SA, disappear into the serum background peak.

The phase system used was found also to be suitable for the analysis of SA in urine. The urine of the subject was collected 2—4 h after drug administration as above. Fig. 4 shows the presence of SA and phenacetin when the urine is injected directly into the column.

The background can be improved by successive extractions of the urine with hexane at pH 10 (discard the hexane layer) and with diethylether at pH 1 (discard the water layer). After evaporation of the diethylether the extract is dissolved in the eluent [15].

Improvement of the resolution of SA and phenacetin can be achieved by modifying the mobile phase (0.05 M $HClO_4$, pH 1.5 instead of 0.25 M $HClO_4$, pH 0.7) [15].

In order to investigate the time course of SA, the serum of a rheumatic patient was examined. The patient was fasted overnight and a blood sample was

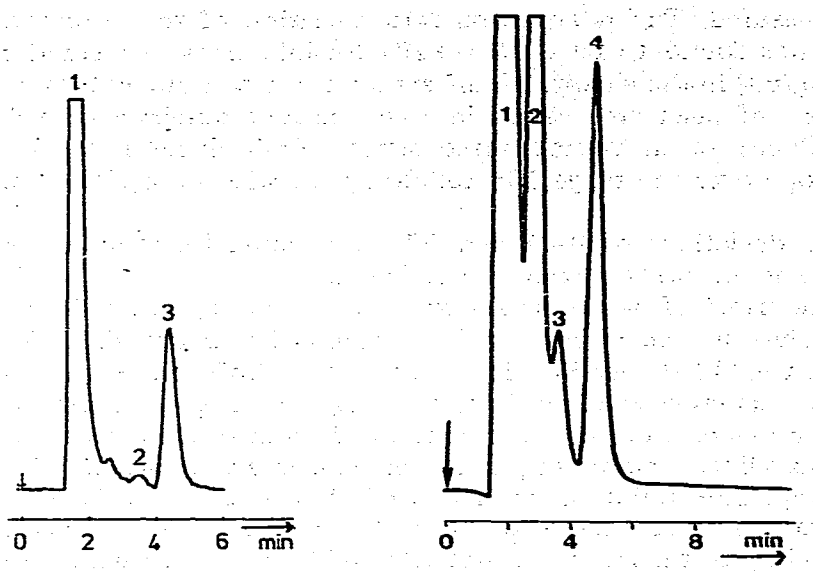

Fig. 3. Chromatogram of deproteinized serum, taken 45 min after oral administration of 200 mg ASA (aspirin), phenacetin, and caffeine. Conditions as in Fig. 2. 1 = Serum background + caffeine + ASA; 2 = phenacetin; 3 = SA.

Fig. 4. Direct injection of urine collected 2—4 h after oral administration of 200 mg caffeine, ASA and phenacetin. Conditions as in Fig. 2. 1 and 2 = Urine background; 3 = phenacetin; 4 = SA.

then taken. Blood samples were subsequently taken at time intervals of ½, 1, 2, 3½, 6 and 8 h after an oral administration of 10 ml medicine containing 1 g sodium salicylate. No heparin or citrate was added to the blood samples before centrifugation (10 min, 300 *g*). In accordance with the results of others [1], an exponential decrease in the SA concentration in serum was found, as demonstrated in Fig. 5. At the most sensitive detector attenuation (0.02 a.u.f.s.) no metabolites of SA could be detected in the chromatograms of the samples. Considering the more polar character of these possible metabolites it might be assumed that they are not retained by the phase system used. If one is interested in particular metabolites, the phase system TOA—Na_2HPO_4—$HClO_4$, pH 5.5 is more suitable as the retention at this pH can be influenced by the anion concentration [14,15].

It was noticed that a higher percentage of SA was found when heparin was added to the blood sample before centrifugation (probably as a result of adsorption of SA at the clod). In blood plasma (heparin added) 8% more SA was found than in serum.

For rheumatic patients, indomethacin is used as a medicine combined with sodium salicylate [6,16]. Recently, HPLC analysis of indomethacin in plasma has been reported [17]. However, SA was left out of consideration. The phase system used in the present paper showed a very large capacity ratio for indomethacin. In order to determine indomethacin and SA simultaneously, column switching or addition of methanol to the eluent [15] is necessary.

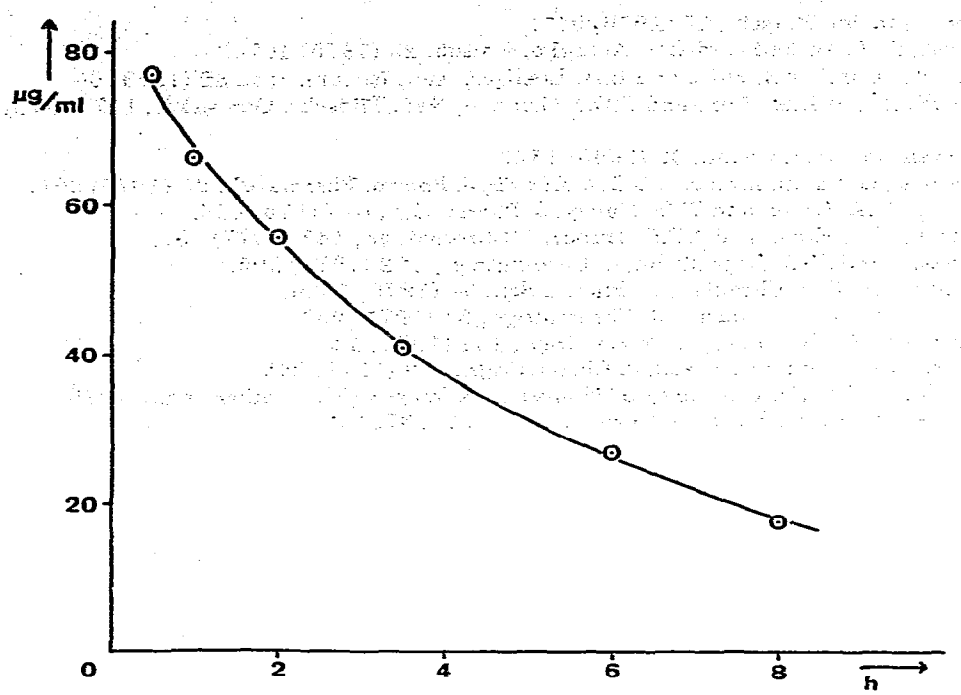

Fig. 5. Time course of SA in serum after oral administration of 1 g sodium salicylate.

CONCLUSIONS

Only a small amount of blood serum is required to determine salicylic acid down to the ppb level by means of direct injection of deproteinized serum into the chromatographic system.

The method is simple and reliable. Many samples can be handled in a short time. The phase system showed remarkable stability and no change in the chromatographic properties was noticed after 2000 injections of deproteinized serum. These qualifications favour routine analysis.

Future research will be devoted to the simultaneous determination of salicylic acid and indomethacin.

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