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DERIVATIZATION OF ENDOGENOUS AND EXOGENOUS COMPOUNDS IN PLASMA FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

S. P. KATRUKHA* and V. G. KUKES
First Medical Institute, Moscow (U.S.S.R.)

SUMMARY

Methods are described for the analysis of endogenous (acetoacetic and pyruvic acids) and exogenous (propranolol) compounds in plasma. Acetoacetic and pyruvic acids were converted into 2,4-dinitrophenylhydrazones and identified by chromatography on a normal-phase silica gel column at 360 nm. The propranolol amino group was modified with dansyl chloride and the dansyl derivative, after extraction with a hydrophobic solvent, was analysed on a normal-phase silica gel column using fluorescence detection. The method allows the determination of 0.5 ng/ml of propranolol.

INTRODUCTION

High-performance liquid chromatography (HPLC), which allows rapid and accurate analyses, is of continuing importance in the medical field. When dealing with biological fluids and tissues in contrast to the assay of standard mixtures and drugs, there are difficulties associated with the nature of the substances and the multi-component systems involved. First, the analysis of hydrophilic substances requires special methods of extraction and preliminary separation of a compound of interest from other biological liquid components. Second, substances with a low molar absorptivity in the UV and visible regions of the spectrum are detected with difficulty, especially at low concentrations. Third, there are particular difficulties with complex compositions of biological samples and it is impossible to single out a compound of interest. Naturally occurring compounds may co-elute and contaminate a carrier in a column, reducing the column lifetime. In our opinion, derivatization of compounds of interest may overcome the above difficulties and simplify analyses. In this study pre-column derivatization of compounds with imino and carbonyl groups was used.

EXPERIMENTAL

Reagents

Lithium acetoacetate was prepared according to Hall[1]. Sodium pyruvate and dansyl chloride (Dns-Cl) were purchased from Merck (F.R.G.) 2,4-Dinitrophenylhydrazine was purified by recrystallization from ethyl acetate. Chloroform was

washed with water and dehydrated with anhydrous calcium chloride before use. Benzene was of spectral grade and acetone and formic acid were of chemical grade.

Chromatography

The chromatography system consisted of a Model 110 A high-pressure pump (Altex, U.S.A.), an injector (with a 20-μl injector loop for keto acids and a 50-μl loop for propranolol) and a normal-phase Partisil PXS 5 silica gel column (250 mm × 4.6 mm I.D.). For the detection of 2,4-dinitrophenylhydrazone (DNP) derivatives of keto acids a Hitachi (Japan) variable-wavelength spectrophotometer was used and for Dns-propranolol detection a Gilson (France) fluorescence detector equipped with an excitation filter at 330–380 nm and a Fluorescamine I emission filter. The flow-rate was 1 ml/min. The column effluent was monitored at a chart speed of 20 cm/h on a Linear 10-mV recorder. The peak heights were measured for quantitative analysis.

Analysis of keto acids present in plasma by HPLC

Equal volumes of plasma and 2,4-dinitrophenylhydrazine solution in 4 *M* hydrochloric acid (50 μg in 100 μl) were shaken for 60 s and then adjusted to pH 2.0–3.0 with 2 *M* sodium hydroxide. The DNP derivatives were extracted from the reaction mixture with 0.8 ml of benzene by vigorous shaking for 3 min. After centrifugation for 3 min at 2000 rpm, 20 μl of the benzene fraction were loaded on to a silica gel column previously equilibrated with mobile phase (chloroform–formic acid, 97.85:2.15). The DNP-keto acids were detected at 360 nm.

Analysis of propranolol in plasma by HPLC

To 1 ml of plasma saturated with sodium carbonate and sodium sulphate was added 2 ml of benzene. The mixture was carefully stirred for 5 min, centrifuged at 2000 rpm and 1 ml of the benzene phase was evaporated to dryness under a stream of nitrogen at 50°C. To the residue were added 100 μl of 0.5 *M* sodium hydrogen carbonate solution and 100 μl of 1% Dns-Cl solution in acetone. The mixture was thermostated for 2 h at 37°C, followed by the addition of 50 μl of Dns-Cl solution, and then incubated again for 2 h at 37°C. Excess of Dns-Cl was removed by adding 200 μl of sodium carbonate solution and incubation for 10 min at 70°C. Dns-propranolol was extracted with 1.5 ml of benzene, centrifuged and 1 ml of the benzene extract was evaporated to dryness under a stream of nitrogen at 50°C. The residue was dissolved in 0.1 ml of mobile phase (chloroform–acetone, 96.9:3.1) and a 50-μl aliquot was injected into the chromatograph.

RESULTS AND DISCUSSION

The concentration of acetoacetic and pyruvic acids in blood as an index of carbohydrate and lipid consumption is of considerable interest. Acetoacetic acid, as other β-keto acids, is very unstable and therefore there are no reliable and sensitive methods for its routine assay in body fluids. For the determination of acetoacetic and pyruvic acids we have developed a procedure using HPLC of the DNP derivatives, which are strongly coloured and have high molar absorptivities at 360 nm[2]. In assays described earlier, DNP derivatives of keto compounds were prepared at ele-

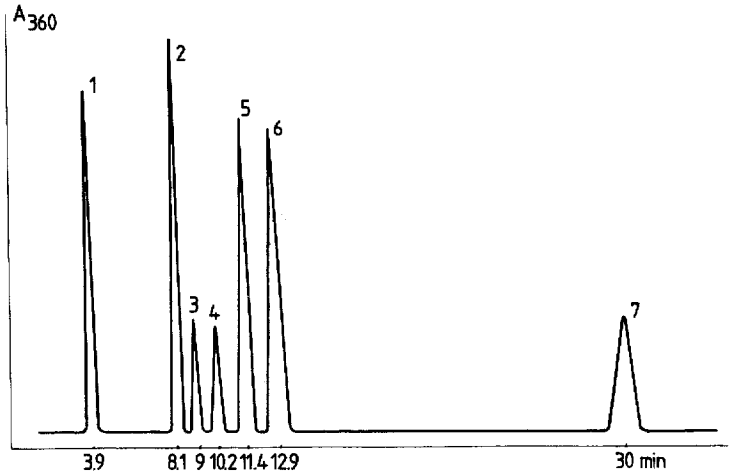

Fig. 1. Chromatogram of a standard mixture of DNP-derivatives of organic keto acids. 1 = Acetone, acetoacetate-1; 2 = acetoacetate-2, α-ketobutyric acid-1; 3 = pyruvate-1; 4 = α-ketobutyric acid-2; 5 = acetylacetone; 6 = pyruvate-2; 7 = α-ketoglutaric acid.

vated temperature in a strongly acidic medium or in an organic solvent[3]. The final products usually consist of a mixture of *cis*- and *trans*-isomers.

As acetoacetic acid is destroyed in acidic medium on heating, we used the saturated 2,4-dinitrophenylhydrazine solution in 2 *M* hydrochloric acid. The reaction was carried out for only 1 min at room temperature, followed by rapid basification to pH 2.0–3.0 with 2 *M* sodium hydroxide solution. When prepared under such conditions, acetoacetic acid DNP gives two peaks on a silica gel column with retention times of 3.9 and 8.1 min (Figs. 1 and 2). The minor peak (acetoacetate-1) (3.9 min) represents not more than 2–3% of the total and its retention time is identical with that of acetone DNP. Pyruvic acid DNP is also formed as a mixture of two substances with retention times of 9 min (pyruvate-1) and 12.9 min (pyruvate-2).

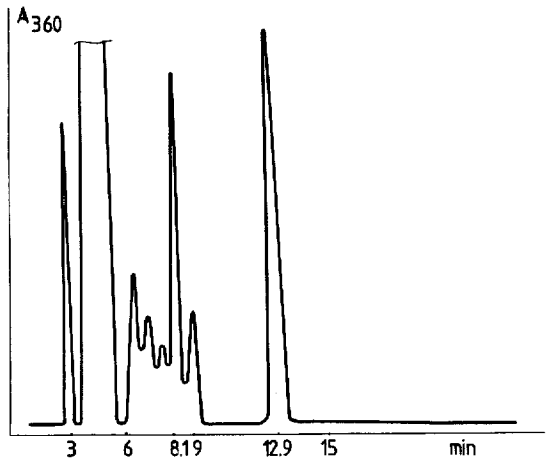

Fig. 2. Determination of acetoacetic and pyruvic acids in plasma.

Pyruvate-1 represents 8–15% of the total. Extraction of DNP derivatives from the reaction mixture was carried out with benzene, as they are less degraded than in diethyl ether, ethyl acetate or chloroform.

The chromatography of keto acid DNP derivatives was carried out on a normal-phase silica gel column with a mobile phase consisting of chloroform–formic acid (97.85:2.15). The presence of formic acid in the mobile phase sharpened the elution peaks and greatly reduced the analysis time. For the determination of acetone in the blood of diabetic patients it is necessary at the end of the reaction to add acetylacetone which reacts with the excess of 2,4-dinitrophenylhydrazine to form a derivative that does not interfere in the keto acid determination. Fig. 1 shows that the acetoacetate-2 peak coincides with one of the α-ketobutyric acid DNP peaks. However, this should not affect the results of acetoacetic acid determination provided that the α-ketobutyric acid concentration in the plasma is extremely small[4]. α-Ketoglutaric acid DNP is not extractable from aqueous medium with benzene, so it was extracted with ethyl acetate and, in order to shorten the assay time, the formic acid concentration in the mobile phase was increased.

The instrument was calibrated in two ways. First, DNP derivatives prepared from pyruvic acid solutions of different concentration were used; second, solutions of analytically pure pyruvic acid DNP were taken as standard solutions. The C/H (concentration/height) deviation from the average was no more than 2% in either method of calibration. The reproducibility of the method was also examined by addition of standard pyruvic acid solutions to plasma. The reproducibility was 96–97% in all instances.

As previously mentioned, acetoacetic acid is extremely unstable. Although lithium acetoacetate was freshly prepared and recrystallized, the purity of this reagent cannot be accepted as 100%. Therefore, the calibration was carried out using only its DNP derivative, the purity of which can be easily examined by chromatography and UV spectrometry. Acetoacetic acid DNP solutions were prepared directly before loading on to the column. The greatest C/H deviation from the average was 2–3%.

The developed method was used for the determination of acetoacetic and pyruvic acid concentrations in the blood of sportsmen during physical exercise. Their respective concentrations in plasma during rest condition were 0.067 ± 0.015 and 0.059 ± 0.022 μmol/ml.

Previously reported methods of keto acid analysis commonly include a stage of preliminary deproteinization with strongly acidic solutions (perchloric, trichloroacetic and hydrochloric acid) following evaporation or neutralization of the supernatant[4,5]. These operations can cause considerable degradation of acetoacetic acid and consequent distortion of the results. The suggested method allows the determination of pyruvic and acetoacetic acid concentrations in plasma avoiding deproteinization. Proteins precipitated after adding 2,4-dinitrophenylhydrazine solution in 4 M hydrochloric acid to plasma do not interfere in the extraction of DNP derivatives with benzene and subsequent separation of the organic and aqueous phases. The analysis takes not more than 30 min.

Propranolol is widely used in the treatment of angina pectoris and hypertension[6,7]. During its determination in plasma, extraction with an organic solvent preceded derivatization[8]. As in the determination of keto acids, the best extraction solvent was benzene. Saturation of plasma with sodium carbonate creates the pH neces-

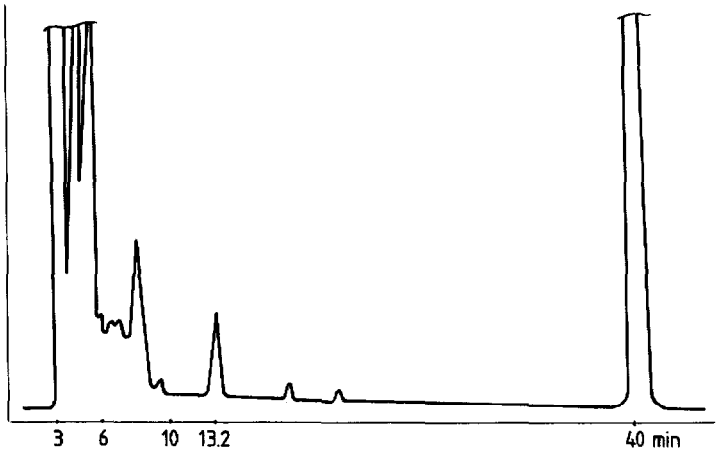

Fig. 3. Chromatogram of an extract of blank serum (without propranolol).

sary for the extraction and a simultaneously high ionic strength of the solution. These conditions ensure almost quantitative extraction of propranolol from plasma. In addition, because of the hydrophobicity of benzene, hydrophilic polar endogenous compounds in plasma are not extracted, which, unlike the previously used ethyl acetate and amyl alcohol, simplifies further analysis and does not contaminate the column.

The Dns-Cl reaction with the second nitrogen atom of propranolol proceeds faster at an elevated temperature (37°C). Further heating of the reaction mixture to 70°C transforms the excess of Dns-Cl into Dns-acid, most of which remains in the aqueous phase. Dns-propranolol is easily separated from Dns-amide and Dns-acid on a normal-phase column using chloroform–acetone (96.9:3.1). Figs. 3 and 4 show typical chromatograms of a blank plasma extract and a plasma sample spiked with propranolol. No interfering peaks are observed. The introduction of the Dns label

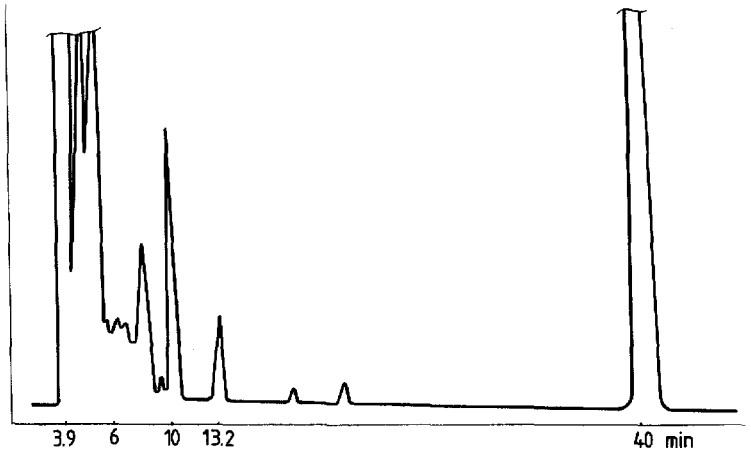

Fig. 4. Chromatogram of an extract of a patient's serum obtained 3 h after oral administration of 80 mg of propranolol.

makes the method highly sensitive, as coupled with fluorescence detection it permits the detection of 0.5 ng/ml of propranolol in plasma. A linear response was observed for concentrations of propranolol ranging from 10 to 500 ng/ml, thus providing a more than adequate range for the determination of propranolol at concentration levels found in plasma.

In conclusion, the proposed methods for derivatization of acetoacetic and pyruvic acids and propranolol with hydrophobic reagents allow (1) complete extraction of hydrophilic compounds from plasma with minimum concomitant compounds; (2) chromatography on normal-phase silica gel, which simplifies operations with the column; and (3) the identification of micro amounts of endogenous and exogenous compounds in small volumes (0.1–1.0 ml) of plasma.

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