Journal of Chromatography, 416 (1987) 237–245
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3567

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SERUM LONG-CHAIN FATTY ACIDS BY DIRECT DERIVATIZATION METHOD

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(First received October 10th, 1986; revised manuscript received December 27th, 1986)

SUMMARY

A new visible–ultraviolet labelling method for the high-performance liquid chromatographic analysis in serum of individual free fatty acids, including polyunsaturated fatty acids, is described. Without commonly used isolation steps, fatty acids in serum were directly derivatized by treatment with acidic 2-nitrophenylhydrazine hydrochloride. The derivatized fatty acids were extracted into *n*-hexane and separated isocratically on a reversed-phase C_8 column within 15 min. The detection limits ranged from 400 fmol to 1 pmol and from 100 to 200 fmol per injection with visible and ultraviolet detection, respectively. Visible detection had better selectivity, and free fatty acid levels were determined in sera obtained from healthy controls and patients with diabetes mellitus. In all the subjects studied, the precise quantitation could be performed with 25 μl of serum. Analytical recoveries ranged from 98.3 to 103.4%. The intra- and inter-assay coefficients of variation were less than 2.7 and 3.5%, respectively. The present method is superior to the previously published methods for routine analyses: it is cheaper, the procedure is simpler, the analysis time is shorter and both resolution and sensitivity are better.

INTRODUCTION

Free fatty acids in blood, derived mainly from triacylglycerols in adipose tissues by the action of hormone-sensitive lipase or from lipoprotein fractions by the action of lipoprotein lipase, play a central role in energy metabolism. The monitoring of individual fatty acid levels in serum or plasma is known to be useful in

0378-4347/87/$03.50

the management of patients with several diseases, such as diabetes mellitus [1], thyremphraxis and hepatic dysfunction [2].

Several high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of these compounds in serum or plasma, employing pre-column derivatization techniques to increase the sensitivity and selectivity of detection [3–8]. One drawback of these methods is that lengthy and cumbersome clean-up procedures, such as liquid–liquid extraction or Extrelut disposable column extraction, are needed for the isolation of the fatty acids from the biological materials prior to the derivatization. Another drawback is that, in spite of a fairly long analysis time (40–70 min), most of these methods [3–6,8] cannot produce simultaneous measurements of biologically important polyunsaturated fatty acids, such as linolenic, dihomo-γ-linolenic, eicosapentaenoic and docosahexanenoic acids. Shimomura et al. [7] achieved the simultaneous determination of these acids in serum, but they required a long analysis time (60 min) and a large volume of serum (0.3 ml). Therefore, the establishment of a simpler and more rapid method is desirable for the screening of large numbers of samples.

We have previously described the determination of fatty acids by reversed-phase HPLC after derivatization with 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1-EDC·HCl) [9–12].

The aims of the present study are to develop a direct derivatization method for the HPLC analysis of fatty acids in serum without conventional isolation steps of these acids, and to examine its analytical utility when applied to clinical investigation.

EXPERIMENTAL

Reagents and chemicals

Oleic ($C_{18:1}$), linoleic ($C_{18:2}$), linolenic ($C_{18:3}$), arachidonic ($C_{20:4}$) acids and 1-EDC·HCl were purchased from Sigma (St. Louis, MO, U.S.A.). Lauric ($C_{12:0}$), myristic ($C_{14:0}$), myristoleic ($C_{14:1}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$) and stearic ($C_{18:0}$) acids were obtained from Japan Chromato Kogyo (Tokyo, Japan). Capric ($C_{10:0}$) and margaric ($C_{17:0}$) acids, pyridine and 2-NPH·HCl were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Dihomo-γ-linolenic ($C_{20:3}$), eicosapentaenoic ($C_{20:5}$) and docosahexaenoic ($C_{22:6}$) acids were purchased from Funakoshi (Tokyo, Japan). Analytical-reagent-grade acetonitrile was obtained from Wako (Osaka, Japan). All reagents and chemicals were used without further purification.

Reagent solution

An aqueous solution of 2-NPH·HCl (20 m*M*) and an acidic solution of 2-NPH·HCl (20 m*M*) were prepared by dissolving the reagent in water and 40 m*M* hydrochloric acid–ethanol (3:1, v/v), respectively. Solutions of pyridine in ethanol (13%, v/v) and of 1-EDC·HCl (250 m*M*) in ethanol were prepared, then a working solution of 1-EDC·HCl was prepared by mixing equal volumes of these

two solutions. Potassium hydroxide (15%, w/v) was dissolved in methanol–water (4:1, v/v).

Derivatization procedure

Fatty acids were converted into their 2-nitrophenylhydrazides as previously described [9–12]. Briefly, to 50 μl of an ethanolic mixture of standard fatty acids, 100 μl of aqueous 2-NPH·HCl solution and 200 μl of working 1-EDC·HCl solution were added, and the mixture was heated at 60°C for 20 min. After the addition of 50 μl of potassium hydroxide solution, the mixture was further heated at 60°C for 15 min, then cooled in running water.

Assay of free fatty acids in human serum

To 25 μl of human serum, 25 μl of ethanol containing 2 nmol of margaric acid as internal standard (I.S.) were added. The serum sample was treated according to the derivatization procedure with acidic 2-NPH·HCl solution. To the resulting mixture of hydrazides 2 ml of 1/30 *M* phosphate buffer (pH 6.4)–0.5 *M* hydrochloric acid (3.8:0.4, v/v) and 1.5 ml of *n*-hexane were added. After vortexing (30 s) and centrifugation (1500 *g*, 5 min), the *n*-hexane layer was taken and evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl of methanol and an aliquot (2–10 μl) was injected into the chromatograph.

Calculation

Previous work had demonstrated that the calibration curves of individual fatty acids were linear over a wide concentration range with good correlation coefficients [11]. Thus, the amounts of individual fatty acids (FA) in serum were calculated according to the equation

$$\text{FA concentration (nmol/ml)} = \frac{\text{peak height of FA}}{\text{peak height of I.S.}} \times \text{amounts of I.S. (nmol)} \times \frac{1000}{\text{sample volume } (\mu\text{l})} \times \text{factor}$$

where factor (standard) is

$$\frac{\text{peak height of I.S.}}{\text{peak height of FA}} \times \frac{\text{amounts of FA (nmol)}}{\text{amounts of I.S. (nmol)}}$$

HPLC analysis

Chromatographic analyses were carried out using a Shimadzu Model LC-5A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with a Shimadzu Model SPD-6AV variable-wavelength visible–UV detector, which was set to monitor the absorbance at 400 nm. Chromatograms were recorded with a multi-pen recorder (Rikadenki Kogyo, Tokyo, Japan). The separation was achieved with a YMC-C8 reversed-phase column (particle size 5 μm, 250×4.6 mm I.D.), packed in Yamamura Chemical Research Institute (Kyoto, Japan). The column temperature was kept constant at 30°C using a Shimadzu GTO-2A

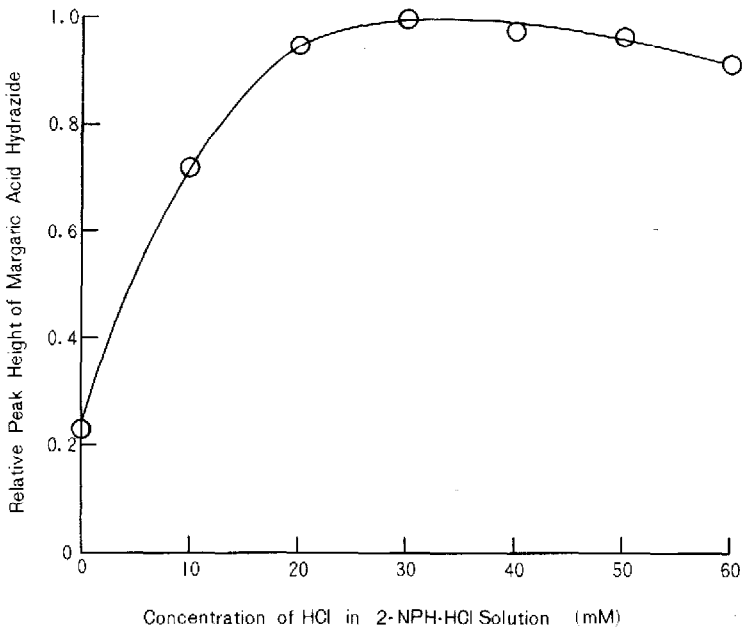

Fig. 1. Effect of the hydrochloric acid concentration in 2-NPH·HCl solution on the peak height of margaric acid; 2 nmol of margaric acid in serum was treated by the assay procedure using various concentrations of hydrochloric acid in 2-NPH·HCl solution.

column oven. All analyses were carried out isocratically using acetonitrile–water (85:15, v/v) as the eluent at a flow-rate of 1.2 ml/min. The pH of the solvent was maintained at 4.5 by adding acetonitrile–0.1 *M* hydrochloric acid (85:15, v/v). The solvent was passed through a Fluoropore filter (pore size 0.45 μm) (Sumitomo Electric, Osaka, Japan) and degassed with a Sonifer-B 12 (Branson Sonic CT, U.S.A.) before use.

RESULTS AND DISCUSSION

We have recently found that fatty acids in aqueous and aqueous ethanolic solutions react sensitively with 2-NPH·HCl using 1-EDC·HCl as a coupling agent to give acid hydrazides [9–12]. We have now used this procedure to investigate the direct derivatization of free fatty acids in serum without complicated isolation steps.

The major problem arising in the direct derivatization of serum fatty acids is the presence of protein-bound acids. Use of ethanol as a precipitating agent for protein makes a convenient method for obtaining profiles of total organic acids in body fluids and tissue homogenates [13–15]. The ethanol content of the reaction mixture in the present method was ca. 75% (v/v). In addition, the mixture was heated at 60°C. Consequently, the protein-bound acids were deproteinized and were scarcely converted into their hydrazides.

During the coupling reaction, the pH of the reaction mixture is one of the principal parameters to ensure the maximum derivatization of the fatty acids. The

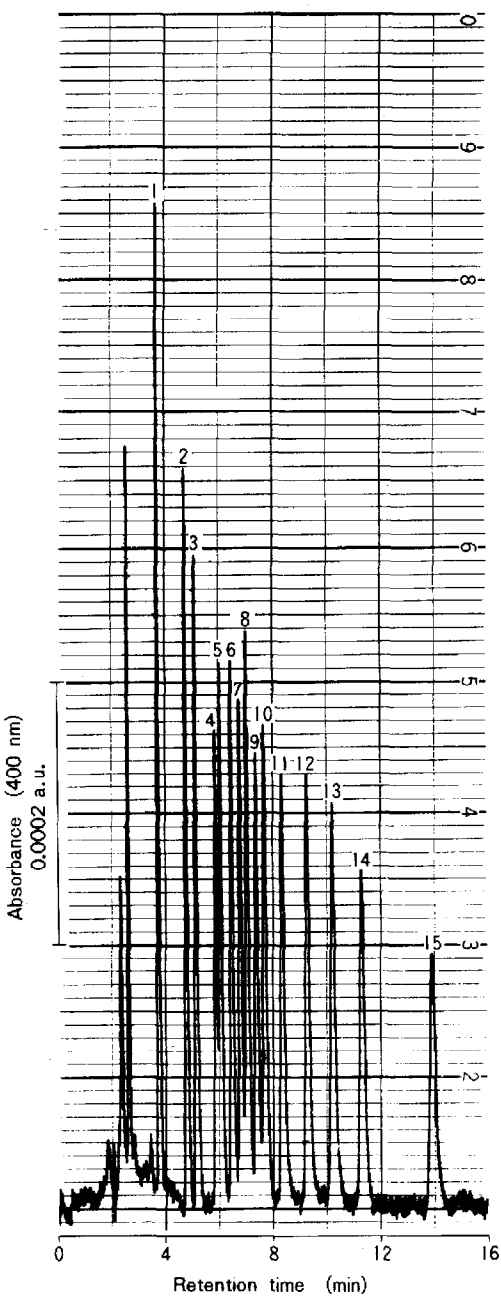

Fig. 2. Chromatogram of the 2-nitrophenylhydrazides of a synthetic mixture of fifteen fatty acids obtained with visible detection. Peaks: 1 = capric ($C_{10:0}$); 2 = lauric ($C_{12:0}$); 3 = myristoleic ($C_{14:1}$); 4 = eicosapentaenoic ($C_{20:5}$); 5 = linolenic ($C_{18:3}$); 6 = myristic ($C_{14:0}$); 7 = docosahexaenoic ($C_{22:6}$); 8 = palmitoleic ($C_{16:1}$); 9 = arachidonic ($C_{20:4}$); 10 = linoleic ($C_{18:2}$); 11 = dihomo-γ-linolenic ($C_{20:3}$); 12 = palmitic ($C_{16:0}$); 13 = oleic ($C_{18:1}$); 14 = margaric ($C_{17:0}$) (I.S.); 15 = stearic ($C_{18:0}$) acid. Each peak corresponds to 10 pmol.

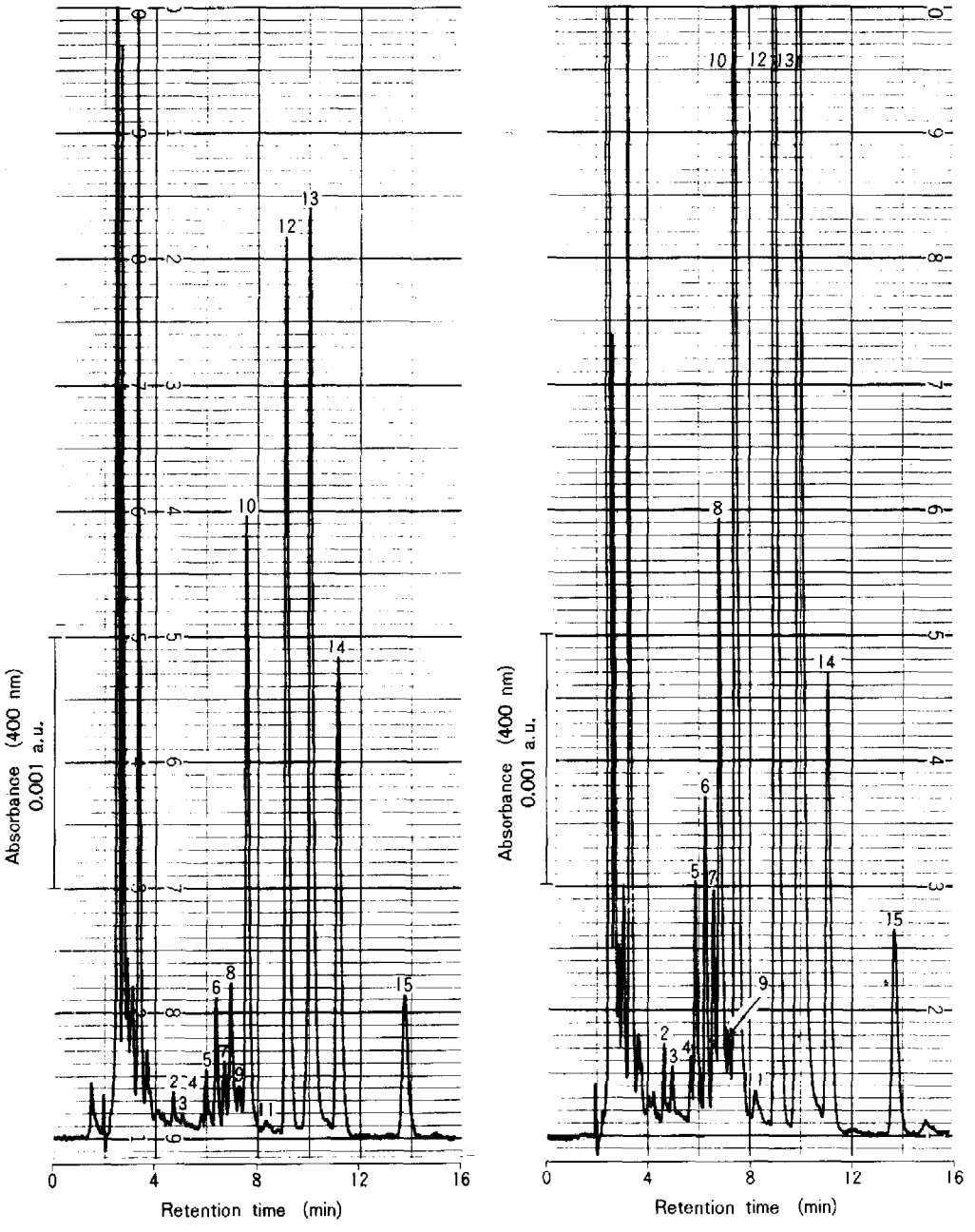

Fig. 3. Free fatty acid profile of serum from a normal subject. Peaks as in Fig. 2.

Fig. 4. Free fatty acid profile of serum from a patient with diabetes mellitus. Peaks as in Fig. 2.

pH of the reaction mixture was slightly increased by the basic substances that occurred in serum samples. This led to a decrease in the yield of the acid hydrazides. The effect of the hydrochloric acid concentration of the 2-NPH·HCl solu-

tion on the peak height of margaric acid hydrazide is shown in Fig. 1. Relatively higher peaks were obtained in the concentration range 20–50 m*M* hydrochloric acid without affecting the interfering peaks, and 30 m*M* was selected for this study.

During the HPLC analysis, the UV detection (230 nm) of capric, lauric and myristoleic acid hydrazides was sometimes interfered with by a few components in the serum matrix, when analysed by the direct derivatization method. This difficulty can be overcome by using visible detection, because the interfering substances do not absorb visible radiation at 400 nm. The advantage of using visible detection is that the chromatograms are simpler and more selective, in spite of approximately four-fold lower sensitive than when UV detection is used in the case of long-chain fatty acid hydrozides. By increasing the detector sensitivity, fourteen fatty acids could be measured in a range from 400 fmol to 1 pmol per injection at a signal-to-noise ratio of 2 with visible detection. On the other hand, UV detection could measure the same samples in the range 100–200 fmol per injection. Fig. 2 shows a chromatogram of the hydrazides of fourteen fatty acids obtained employing visible detection with margaric acid as I.S. Each peak corresponds to 10 pmol of the fatty acid.

Long-chain fatty acids are present in serum principally in esterified form with triacylglycerols and glycerophospholipids. Hatsumi et al. [16] demonstrated that some fatty acids, such as $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, were remarkably released from phosphatidylinositol during the commonly used process of chloroform extraction. To examine whether the fatty acids of these glycerolipids were derivatized, triglycerides, L-α-phosphatidyl-DL-glycerol, L-α-phosphatidylcholine and L-α-phosphatidylserine (1 mg in each sample) were derivatized as described above. Under the reaction conditions, no fatty acids were observed in the chromatograms.

Recovery and reproducibility were investigated six times by adding a known mixture of fourteen fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ 1 nmol each, others 0.3 nmol) to 25 μl of human serum. The recoveries of the fatty acids were in the range 98.3–103.4% and the coefficients of variation were in the range 0.7–3.1%. The intra-assay precision was evaluated by assaying six times the same human serum sample. The inter-assay precision was determined by assaying spiked human serum on different days over one week ($n=6$). The intra- and inter-assay coefficients of variation were less than 2.7 and 3.5%, respectively. These results indicate that the present method has a satisfactory precision in analysing fatty acid levels in serum.

To confirm the clinical utility, serum samples from sixteen fasting normal subjects (ten men and six women) and 21 fasting patients with non-insulin-dependent diabetes mellitus (eight men and thirteen women) were similarly analysed. The fatty acid profiles of serum samples from a normal control and a diabetic subject are shown in Figs. 3 and 4, respectively. The chromatograms monitored by visible absorbance show a very clean visible background, and thus the fatty acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards.

Comparison of the present method with the previously published method [12]

TABLE I

FREE FATTY ACID COMPOSITIONS OF SERUM SPECIMENS FROM NORMAL AND DIABETIC SUBJECTS

Data are expressed as the mean ± S.D. The significant difference between the two groups was assessed using the Student's *t*-test.

Fatty acid	Free fatty acid composition (mol %)	
	Normal ($n=16$)	Diabetic ($n=21$)
$C_{12:0}$	0.81 ± 0.48	0.69 ± 0.24
$C_{14:0}$	3.32 ± 1.31	3.14 ± 0.51
$C_{14:1}$	0.36 ± 0.13	0.42 ± 0.27
$C_{16:0}$	27.13 ± 2.19	27.52 ± 2.18
$C_{16:1}$	3.49 ± 0.81	4.29 ± 1.53
$C_{18:0}$	7.91 ± 1.58	6.38 ± 1.48*
$C_{18:1}$	30.93 ± 3.29	29.89 ± 1.96
$C_{18:2}$	19.18 ± 1.83	20.16 ± 2.52
$C_{18:3}$	1.77 ± 0.30	2.13 ± 0.45*
$C_{20:3}$	0.36 ± 0.10	0.39 ± 0.16
$C_{20:4}$	1.79 ± 0.51	1.34 ± 0.22**
$C_{20:5}$	0.66 ± 0.33	0.87 ± 0.30
$C_{22:6}$	2.29 ± 1.07	2.77 ± 0.60
Unsaturated fatty acids	60.82 ± 4.00	62.27 ± 3.44
Total concentration (nmol/ml)	321.26 ± 95.41	813.07 ± 280.03***

*$p<0.01$.
**$p<0.005$.
***$p<0.001$.

was made for nine serum samples taken randomly from the two groups. The correlation coefficients between the two methods were in the range 0.982–0.997 for thirteen fatty acids. Furthermore, the results obtained by the present method were compared with those derived by another published method, the 9-anthryldiazomethane (ADAM) method [7], for the nine samples. The correlation coefficients of 0.980–0.994 were satisfactory for twelve acids but not for the $C_{14:1}$ acid, which could not be measured by the ADAM method.

The total concentations and the compositions of free fatty acids in the sera from normal subjects and patients with diabetes mellitus are listed in Table I. The data for the individual free fatty acids in normal sera were in good agreement with those in other reports [6–8,12]. The mean values for the total concentration of free fatty acids was more than 2.5-fold higher in the patients with diabetes mellitus than in the normal subjects. In such patients, the compositions of stearic and arachidonic acids were markedly decreased ($p<0.01$ and $p<0.005$, respectively), whereas linolenic acid was significantly increased ($p<0.01$). There was a tendency for the compositions of palmitoleic, eicosapentaenoic and docosahexaenoic acids in the patients to increase, but without reaching statistical sig-

nificance. The ratio of unsaturated to saturated fatty acids did not differ significantly between the two groups. Further studies are necessary to investigate whether such differences are of clinical significance and are correlated with other parameters, such as blood sugar, glycosylated hemoglobin and ketone bodies.

In conclusion, the present method has two important advantages over previously published methods [3–8,12]. First, the serum fatty acids are directly derivatized in the simple procedure without commonly used extraction and purification steps. This technique reduces the overall analysis time and cuts the required sample volume to only 25 μl. The second advantage is that visible detection can be used, so that interfering peaks do not appear in the chromatograms, and the detection and separation of the peaks for all the fatty acids are performed within 15 min.

Despite the very simple procedure, more than 1000 analyses were made in our laboratories without any deterioration of the separation capability. Therefore, the present method may be successfully used to screen large numbers of sera, because of its great simplicity, saving much time, cost and labour.

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