triglycerides, the oxidation of the released fatty acids may account for an important fraction of the increase in respiration induced by gastric secretagogues. We[8] have shown in a previous study that the stimulatory effects of gastric secretagogues on respiration and acid secretion can be blocked by selective inhibition of fatty acid oxidation. Exogenous fatty acids significantly stimulate respiration and acid secretion, whereas glucose, pyruvate and lactate have little or no effect[10]. In addition, tracer studies have indicated a metabolic preference for fatty acid oxidation in the amphibian gastric mucosa[7,8]. The mechanism of lipid mobilization is not known at present. One possibility is that secretagogues, via cAMP, might activate a lipase in the gastric mucosa. Activation of a lipase by cAMP has been demonstrated in adipose tissue[15]. This point must await further investigation to be elucidated.

1 This investigation was supported by Consejo de Desarrollo Científico y humanistico de la Universidad del Zulia, Maracaibo, Venezuela.
2 To whom correspondence should be addressed.

3 Davenport, H.W., Fedn Proc. *11* (1952) 715.
4 Forte, J.G., and Davies, R.E., Am. J. Physiol. *206* (1964) 218.
5 Alonso, D., and Harris, J.B., Am. J. Physiol. *208* (1965) 18.
6 Berglindh, T., Helander, H.F., and Öbrink, K.J., Acta physiol. scand. *97* (1976) 401.
7 Hersey, S.J., Gastroenterology *73* (1977) 914.
8 Chacín, J., Martínez, G., and Severin, E., Am. J. Physiol. *238* (1980) G255.
9 De Bohórquez, E., and Chacín, J., Experientia *36* (1980) 1198.
10 Alonso, D., Nigon, K., Dorr, I., and Harris, J.B., Am. J. Physiol. *212* (1967) 992.
11 High, W.L., and Hersey, S.J., Am. J. Physiol. *226* (1974) 1408.
12 Hersey, S.J., Biochim. biophys. Acta *244* (1974) 157.
13 Hersey, S.J., in: Gastric hydrogen ion secretion, p.117. Eds D. Kasbekar, G. Sachs and W. Rehm. Dekker, New York 1976.
14 Wieland, O., in: Methods of enzymatic analysis, p.211. Ed. H.U. Bergmeyer. Academic Press, New York 1965.
15 Corbin, J.D., Reiman, E.M., Walsh, D.A., and Krebs, E.G., J. biol. Chem. *245* (1970) 4849.

0014-4754/85/030358-02$1.50 + 0.20/0

Demonstration of retinoic acid isomers in human urine under physiological conditions

W.E. Lambert[1] and A.P. De Leenheer[2]

Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Gent (Belgium), 20 February 1984

Summary. Untransformed retinoic acid has never been demonstrated in human excreta under normal physiological conditions. We have developed a two-step liquid chromatographic system for the demonstration of subnanogram amounts of this compound in human urine without administration of any precursor.
Key words. Retinoic acid metabolites; human urine; high performance liquid chromatography; retinoic acid isomers; UV detection.

The metabolism and excretion of vitamin A has been the subject of numerous investigations. Retinol is primarily metabolized to a number of compounds with an intact side chain. Some of these metabolites were identified as retinoic acid[3], epoxyretinoic acid[4], 4-hydroxy- and 4-ketoretinoic acid[5-7], retinoyl-beta-glucuronides[8,9] and some cyclisized derivatives[10]. Other minor metabolites lack either the terminal carbon or carbons 14 and 15[11,12]. However, in all these experiments large and nonphysiological amounts of retinoic acid itself, retinyl acetate or other structurally related compounds had been administered. Hence the possibility remains that these metabolites do not occur under normal physiological conditions[13]. Retinoic acid is formed by oxidation of the terminal alcoholic group of retinol. The concentration of vitamin A acid in human serum is in the low nanogram range[14,15]. In view of the high biological activity of this compound[16,17], the aim of this study was to look for the presence of retinoic acid in human urine under normal physiological conditions. Due to its nondestructive character and to its high separating capacity, high performance liquid chromatography is the method of choice for this purpose. We describe the isolation and purification of retinoic acid from crude urine extracts.

Materials and methods. All-trans-retinoic acid was purchased from Fluka AG (Buchs, Switzerland). 9-Cis- and 13-cis-retinoic acid were supplied by Hoffmann-La Roche Inc., Nutley (NJ, USA). Reagents and solvents were of analytical grade and were purchased from Merck AG (Darmstadt, West Germany). Isomerization and/or photodegradation of the retinoic acid was avoided by working in a darkened room under yellow light. Whenever possible, low actinic (amberized) glassware was used. Extraction and storage of the organic layer before evaporation were carried out at 4 °C.

Urine samples were from healthy fasting adults (male or female) and the samples were analyzed directly after collection. A pre-extraction followed by a double-phase extraction at different pH values was applied to 200 ml of human urine. At an alkaline pH (10 ml of 2N NaOH), 200 ml of urine together with 100 ml of ethanol were extracted with 400 ml of n-hexane at 4 °C. The n-hexane layer was discarded and a second extraction with another 400 ml n-hexane was performed in acidic medium (15 ml of 2N HCl). The organic phase was concentrated on a Büchler Evapo-Mix (Büchler Instruments Inc., Fort Lee, NJ, USA) or alternatively, under a stream of nitrogen. The residue of an extract of 200 ml of urine was dissolved in 2.2 ml of the chromatographic solvent and two 1-ml aliquots were injected in the chromatographic system. Each sample was centrifuged prior to HPLC analysis to avoid the injection of particulate matter.

The analytical high performance liquid chromatographic system consisted of a Pye Unicam LC_3-XP (Cambridge, England) pump, a sampling valve (Model CV-6-UHPa-N60, Valco Instruments Co., Houston, TX, USA) with a 50-µl loop and a Pye Unicam LC_3-UV variable wavelength detector set at 350 nm and used at the maximum sensitivity (0.005 AUFS).

The analytical column (15 × 0.32 cm) was packed in our laboratory with silica RSIL 5 µm (RSL; St. Martens-Latem, Belgium). Elution was performed with a mixture of n-hexane:acetonitrile:acetic acid (99.5:0.2:0.3, v/v) at a flow rate of 0.75 ml/min.

Extracts of a higher sample volume (200 ml) were subjected to a clean-up step on a semipreparative reversed-phase liquid chromatographic column. A RSIL C18 10 µm column (50 × 1 cm ID) (Alltech Europe, Eke, Belgium) was eluted with methanol:water:acetic acid (89.7:10:0.3, v/v) at a flow rate of 4.5

ml/min. A precolumn (10 × 0.32 cm ID) filled with RSIL C18 30 µm was placed between the injection device and the column to prevent clogging of the semipreparative column. Regenerating of the precolumn with methanol was necessary after about 15 injections, and after three to four washings refilling (dry) of the column was necessary.

Additional evidence for the peak identities was obtained by diazomethane treatment of fractions, corresponding to the elution position of the different retinoic acid isomers, trapped from the C18 column. The derivatized extract was rechromatographed on the silica column and the retention data of the peaks were compared with those of standard compounds of mehtylretinoate isomers.

Results and discussion. Initially, crude extracts of 3.5 ml of urine were directly analyzed on the analytical silica column. From these chromatograms no conclusions could be drawn with regard to the presence or absence of retinoic acid, as a number of compounds elute very close to the position of all-trans-retinoic acid. A purified extract free from interfering substances was thus required, and the procedure had to be scaled up. To this end we submitted a larger amount of human urine to the pre-extraction at alkaline pH, followed by the double phase extraction at acidic pH. This extract was purified on a semipreparative reversed phase column and the fractions corresponding to the elution volume of the retinoic acid isomers were collected. After extraction of these collected fractions with n-hexane we chromatographed them on the analytical silica column. A chromatogram obtained on a 50-µl aliquot, corresponding to 40 ml of urine, is shown in figure 1. Peaks 1, 2 and 3 show the same retention times as 13-cis-, 9-cis- and all-trans-retinoic acid. They are well separated from interfering compounds. Additionally, we re-collected the individual peaks from the silica column. After treatment with diazomethane we reinjected the fractions in the same silica system (fig. 2). This resulted in a clear-cut separation of three peaks in a chromatogram free from interfering substances. Their retention times are identical with those of the different isomers of methylretinoate. The diazomethane treatment not only yielded cleaner chromatograms but also provided additional evidence for the identity of the peaks.

Isomerization of the compounds during sample pre-treatment was prevented by working in the absence of daylight. The chromatographic process itself did not result in the formation of isomers. This was demonstrated by taking a urine sample, supplemented with the all-trans-isomer throughout the whole procedure. This resulted in an increase of the peak of all-trans-retinoic acid while the peaks of the other isomers remained unchanged, indicating that there was no isomerization during the extraction and chromatography. Figure 2 suggests that in urine the predominant form of retinoic acid is the 13-cis-isomer. 9-Cis- and all-trans-retinoic acid are present only in minor quantities. This is in agreement with previous data indicating that the 13-cis-isomer predominates under normal physiological conditions, whereas pharmacological doses of retinoic acid appear to favor excretion of the all-trans form[13]. Indeed, following the administration of pharmacological doses of all-trans-retinoic acid, liver enzymes are overloaded and the excess of retinoic acid is excreted via the alternative trans-route.

For exact quantitative determination of the urinary retinoic acid level the use of an internal standard is highly recommended. Our aim, however, was to demonstrate the presence of retinoic acid in human urine under normal physiological conditions.

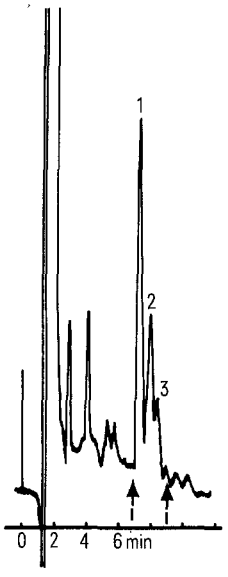

Figure 1. The urinary extract fraction trapped from the semipreparative column, chromatographed on the analytical silica column. Peak identification: 1: 13-cis-, 2: 9-cis-, 3: all-trans-retinoic acid.

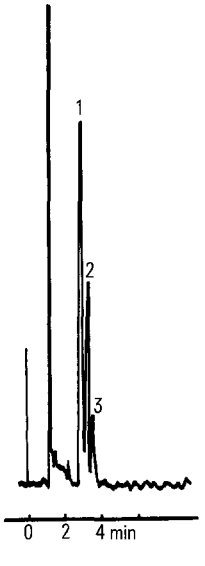

Figure 2. Chromatographic run of a collected fraction from the silica system. The fraction was treated with diazomethane and reinjected on the analytical silica column. Peak identification: 1: 13-cis-, 2: 9-cis-, 3: all-trans-methyl retinoate.

1 The work was supported by a grant of the N.F.S.R. to W.L.
2 To whom all correspondence should be addressed.
3 Ito, Y.L., Zile, M., Ahrens, H., and DeLuca, H.F., J. Lipid Res. *15* (1974) 517.
4 McCormick, A.M., Napoli, J.L., Schnoes, H.K., and DeLuca, H.F., Archs Biochem. Biophys. *192* (1979) 577.
5 Frolik, C.A., Roberts, A.B., Tavela, T.E., Roller, P.P., Newton, D.L., and Sporn, M.B., Biochemistry *18* (1979) 2092.
6 Frolik, C.A., Roller, P.P., Roller, A.B., and Sporn, M.B., J. biol. Chem. *255* (1980) 8057.
7 Hanni, R., and Bigler, F., Helv. chim. Acta *60* (1977) 881.
8 Lippel, K., and Olson, J.A., J. Lipid Res. *9* (1968) 168.
9 Zile, M.H., Schnoes, H.K., and DeLuca, H.F., Proc. natl. Acad. Sci. USA *77* (1980) 3230.
10 Hanni, R., Bigler, F., Meister, W., and Englert, G., Helv. chim. Acta *59* (1976) 2221.
11 Roberts, A.B., and DeLuca, H.F., Biochem. J. *102* (1966) 600.
12 Sundaresan, P.R., and Bhagavan, H.N., Biochem. J. *122* (1970) 1.
13 Swanson, B.N., Frolik, C.A., Zaharevitz, D.W., Roller, P.P., and Sporn, M.B., Biochem. Pharmac. *30* (1980) 107.
14 De Ruyter, M.G., Lambert, W.E., and De Leenheer, A.P., Analyt. Biochem. *98* (1979) 402.
15 De Leenheer, A.P., Lambert, W.E., and Claeys, I., J. Lipid Res. *23* (1982) 1362.
16 Jetten, A.M., and Jetten, M.E.R., Nature *278* (1979) 180.
17 Shapiro, S.S., and Poon, J.P., Exp. Cell Res. *119* (1979) 349.

0014-4754/85/030359-02$1.50 + 0.20/0