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Urinary Steroids from a Newborn Human Infant. Identification of 2α-Hydroxy-4-pregnene-3,20-dione, 3β,15β-Dihydroxy-5-pregnen-20-one and 3β,15α-Dihydroxy-5-pregnen-20-one

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Urinary steroids from healthy newborn human infants were analyzed by gas–liquid chromatography and gas–liquid chromatography-mass spectrometry. The identification of 2α-hydroxy-4-pregnene-3,20-dione and the characterization of its 2β-isomer is recorded here for the first time. Mass spectrometric evidence supporting the identification of 3β,15β-dihydroxy-5-pregnen-20-one and 3β,15α-dihydroxy-5-pregnen-20-one is also presented. Furthermore, the following 15-hydroxylated steroids were also found and identified: 3β,15ξ,16ξ-trihydroxy-5-androsten-17-one, 5-androstene-3β,15α,16α,17β-tetrol, 3β,15β,17-trihydroxy-5-pregnen-20-one and 5-pregnene-3β,15ξ,17,20ξ-tetrol. The origin of these 2- and 15-hydroxylated urinary steroids is discussed in relation to current knowledge of 4-pregnene-3,20-dione and 3β-hydroxy-5-pregnen-20-one metabolism during the human perinatal period.

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INTRODUCTION

Recently, we described the gas–liquid chromatography (GLC) and gas–liquid chromatography-mass spectrometry (GLC-MS) identification of 2α-hydroxy-4-pregnene-3,20-dione and other related compounds in human late-pregnancy urine [1]. It was postulated that the fetal liver could be the main site of 2α-hydroxylation of placental 4-pregnene-3,20-dione. However, the maternal and/or placental compartments have not been excluded as possible sources of this metabolite. Keeping this in mind and, since it is commonly accepted that the steroid metabolism of a human fetus at full-term is comparable to that of a newborn, particularly in the first day of extra-uterine life, we have undertaken to investigate the urinary steroids of healthy newborn human infants, aged 1 day old. The present paper describes the identification of 2α-hydroxy-4-pregnene-3,20-dione and of its possible isomer *viz*, 2β-hydroxy-4-pregnene-3,20-dione in the urine of newborn human infants. Since the isolation and the identification of 15α-hydroxyestrone [2], several 15-hydroxylated steroids have been found in different biological materials ([3–7] and ref. therein). For example 15α-hydroxyestriol has been shown to be a valuable marker of fetal viability during pregnancy [3]. More recently, the identification of some urinary 15β-hydroxylated metabolites of 17-hydroxy-4-pregnene-3,20-dione has been highlighted as an excellent marker for the early diagnosis of the most common form of congenital adrenal hyperplasia. This amounts to a 21-hydroxylase deficiency in the neonatal period [5–7]. In human pregnancy, it is widely accepted that fetal liver is the main site of steroid hydroxylation at C-15 [4, 8–10]. 3β,15α-Dihydroxy-5-pregnen-20-one has been shown to be a normal urinary metabolite of human late-pregnancy [11]. In this study, we also report the tentative identification by GLC-MS of 3β,15β-dihydroxy-5-pregnen-20-one and 3β,15α-dihydroxy-5-pregnen-20-one in the urine of one of the newborns.

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EXPERIMENTAL

Chemicals and reagents

The reference standard of 2α-hydroxy-4-pregnene-3,20-dione was purchased from Steraloids Inc. (Wilton, NH, U.S.A.). Sodium acetate of Normapur quality and glacial acetic acid of analytical grade were obtained from Prolabo (Paris, France) and PANREAC (Madrid, Spain), respectively. Chloroform, methanol, pyridine and *n*-heptane (all analytical grade) were obtained from Carlo Erba (Rueil Malmaison, France). Sep-Pak C_{18} cartridges purchased from Millipore S.A. (St-Quentin-en-Yvelines, France) were primed with 5 ml of methanol and rinsed with 5 ml of distilled water. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). *N-O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and methoxyamine hydrochloride were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) and Eastman Kodak Co. (Rochester, NY, U.S.A.), respectively. *Helix pomatia* digestive juice containing both 100,000 Fishman units (FU) of β-glucuronidase and 1,000,000 Roy Units (RU) of sulfatase per ml was obtained from Reactifs IBF (Villeneuve-la-Garenne, France).

Urine processing

Urine samples (24-h) were obtained from three healthy boys, both aged 1 day old. All the newborn human infants were delivered from normal full-term pregnancies. Urine specimens collected were frozen and stored at − 20°C until analyzed. In each case, the whole amount of the 24-h urine samples was used for analysis of urinary steroids. After centrifugation, filtration and adjusting the pH to 5.20 with 20% acetic acid, sodium acetate buffer (2 M, pH 5.20) and *Helix pomatia* digestive juice (1500 FU of β-glucuronidase and 15,000 RU sulfatase per ml of urine) were added and the urinary mixture was incubated at 37°C for 24 h. Thereafter, a second addition of enzyme was made and the mixture was incubated for a further 24 h. After hydrolysis, free and deconjugated steroids were recovered by solid-phase extraction on Sep-Pak C_{18} cartridges [12]. The cartridges were rinsed with 5 ml of distilled water and the elution of the urinary steroids was carried out with 2 × 5 ml of methanol. The two 5 ml fractions were combined and evaporated under a stream of N_2 at 60°C. The dry steroid residue was dissolved in 0.2 ml of chloroform–heptane–methanol (5:5:1, by vol) mixture and chromatographed on Sephadex LH-20 using a 30 × 0.4 cm i.d. column, as described previously [13]. Elution of the steroids was carried out using chloroform–heptane–methanol (5:5:1, by vol) as a solvent system. Sixty fractions of 1 ml were collected. Samples from each fraction were analyzed for steroid content by GLC and GLC-MS as the TMS ethers and MO-TMS derivatives. The TMS ethers were prepared by adding 150 μl of pyridine–BSTFA (1:3, v/v) to the dry steroid residue and heated at 60°C overnight. The MO-TMS derivatives were obtained by adding 200 μl of methoxyamine hydrochloride reagent to pyridine (16 mg/ml) and this was heated overnight at 60°C. After removing the reagent under a stream of N_2, the trimethylsilylation was performed as described above.

GLC and GLC-MS analysis

GLC was carried out on a Packard 427 GC equipped with a flame-ionization detector and an all-glass solid injector. A fused-silica capillary column (25 m × 0.32 mm i.d.) coated with a 0.33 μm layer of SE-30 was used. Separation was achieved by temperature programming from 180 to 280°C at 2°C/min. The helium carrier gas pressure was set at 1.2 bar and the injection port and detector temperatures at 270 and 298°C, respectively. GLC-MS was performed on a Hewlett–Packard (HP) GC model 5890 interfaced to a HP-5971 A Mass Selective Detector. An HP-1 (cross-linked methyl silicone) fused-silica capillary column (12 m × 0.20 mm i.d., 0.33 μm film thickness), was inserted directly into the ion source. Samples were introduced by splitless injection at an initial oven temperature of 70°C and a purge time of 1 min was used. After 1 min delay, the oven temperature was then programmed to 200°C at 25°C/min and to 300°C at 2°C/min. Other experimental conditions were: helium carrier gas pressure, 0.9 bar; injector and transfer line temperatures, 250 and 300°C, respectively; energy of bombarding electrons, 70 eV; Electron Multiplier Voltage, 1800–1900 V. Mass spectra were obtained by repetitive scanning of the m/z range 100–650. The mass spectrometer was also used in selected ion monitoring (SIM) mode. A compound was considered identified when it showed the chromatographic and mass spectrometric data, which were identical to those of an authentic standard or of a compound published previously, when the reference standard was not available.

RESULTS

The analysis by GLC-MS of the steroids eluted in Sephadex LH-20 fraction 2 revealed the presence of two compounds (compound **I** and **II**) which showed, both as TMS ether and MO-TMS derivative, similar mass spectra to those obtained with the reference standard of 2α-hydroxy-4-pregnene-3,20-dione (Figs 1 and 2). As discussed previously [1, 14], their MO-TMS mass spectra showed a molecular ion (M) at m/z 460 and typical fragments at m/z 445 (M-15), 429 (M-31, base peak), 370 (M-90), 339 [M-(31 + 90)] and 100 (Fig. 2). Of these fragments, the peak at M-58 (m/z 402) has been proposed to be indicative of the α-trimethylsilyloxy-3-methoxyimino-4-ene steroids [15]. Due to very small amounts of compound **I**, it has not been possible to obtain a completely pure MO-TMS

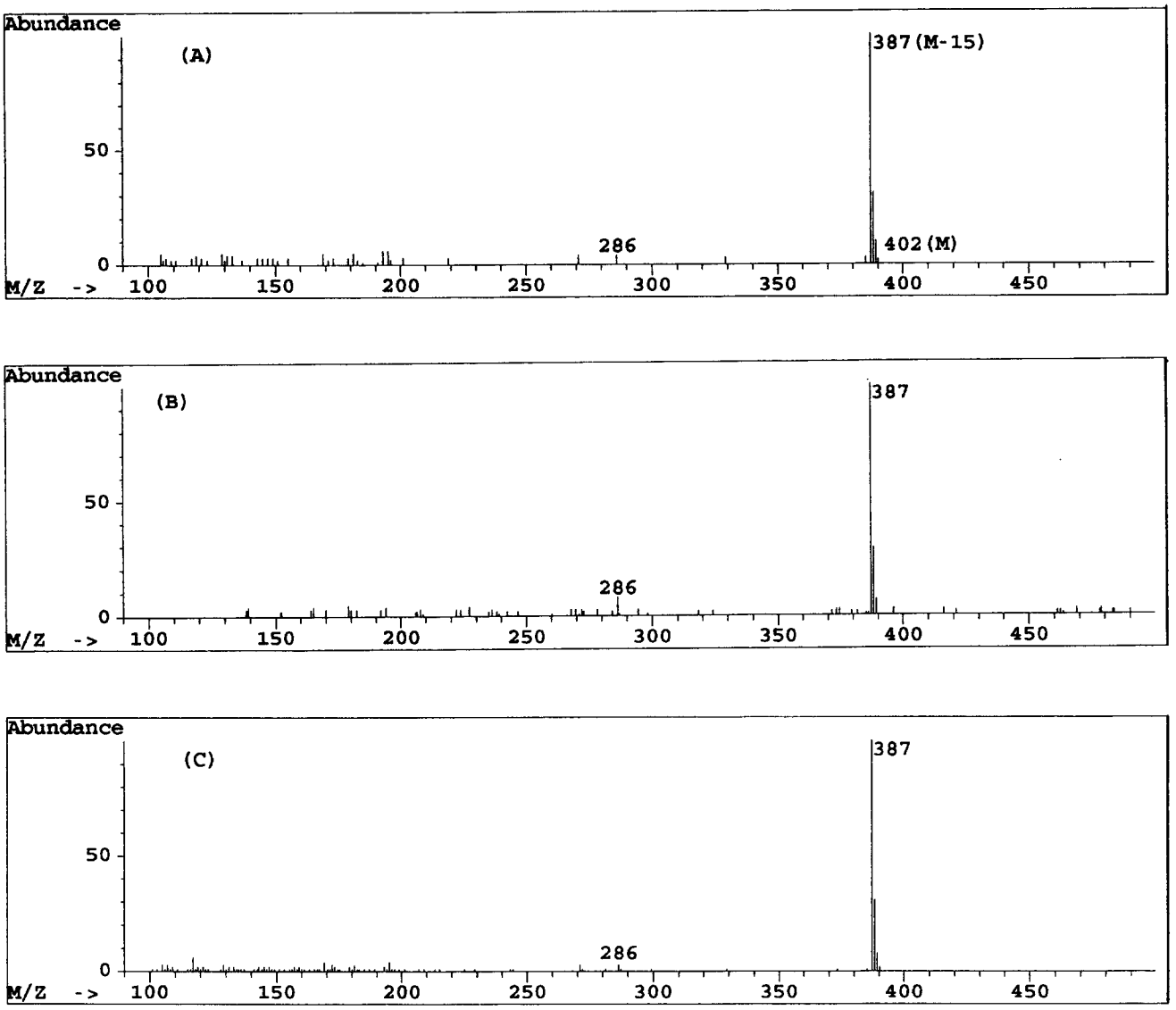

Fig. 1. Mass spectra of the TMS ethers of reference 2α-hydroxy-4-pregnene-3,20-dione (A), compound I (B) and II (C).

mass spectrum. The mass spectra of the TMS ethers of these two compounds given in Fig. 1 were dominated by the peak at *m/z* 387 (M-15). The fragment at *m/z* 286 probably represents the M-116. The loss of 116 mass units from molecular ion is a diagnostically useful feature of 1- and 2-trimethylsilyloxy-3-oxo-4-ene steroids [16, 17]. However, it has previously been found that the fragment at *m/z* 286 is normally the base peak in the TMS ether mass spectrum of 1β-hydroxy-4-pregnene-3,20-dione [18]. More widely, it has previously been observed that the mass spectra of the TMS ethers of several 1β-trimethylsilyloxy-3-oxo-4-ene steroids show the base peaks at M-116, a characteristic which is not shared by their corresponding 2-trimethylsilyloxy-3-oxo-4-ene steroids [16, 18, 19]. In the comparison of the chromatographic properties of compounds I and II (Table 1), only one of these *viz*, compound II, presented identical chromatographic behaviors to those of the reference 2α-hydroxy-4-pregnene-3,20-dione. Based on these data, compound II was identified as the 2α-hydroxy-4-pregnene-3,20-dione. It is interesting to observe that the lower methylene unit (MU) values, both as TMS ethers and MO-TMS derivatives, found in this study for compound I (Table 1) from the corresponding values of the reference 2α-hydroxy-4-pregnene-3,20-dione also corresponded closely with the previous data of the authentic 2β- and 2α-hydroxy-4-pregnene-3,20-dione [20]. Based on these results, compound I was tentatively identified as 2β-hydroxy-4-pregnene-3,20-dione. Finally, due to small amounts of these metabolites, the GLC-MS repetitive scanning process was completed by analysis in SIM mode. The results obtained were also in total agreement with those presented above. The MO-TMS diagram obtained is depicted in Fig. 3.

Table 1. Gas chromatographic characteristics obtained during GLC-MS analyses of the TMS ethers and MO-TMS derivatives of the urinary compounds I, II, III and IV identified in Sephadex LH-20 fractions

	MU		UM_{SIM}	
Compound	TMS	MO-TMS	TMS	MO-TMS
Compound **I**	28.33[a]	28.36[a,b]	28.40[a]	28.33[a]
Compound **II**	28.90	29.13	28.91	29.15
Compound **III**	27.73	27.86	27.72	27.83
Compound **IV**	28.20	28.77	28.17	28.74

MU values were determined during GLC-MS analyses (on HP-1 column) in relation with the C_{24} and C_{32} *n*-alkanes analyzed under the same conditions. For materials and methods see text.

[a]It should be pointed out that due to the very small amount of this product, it was not possible to calculate rigorously its MU.

[b]Chromatographic peak containing several compounds.

Figure 4 displays the total ion current (TIC) chromatogram from GLC-MS analysis of MO-TMS derivative steroids eluted by Sephadex LH-20 fraction 14. Figure 5 displays the MO-TMS mass spectra of the compounds indicated **III** and **IV**. Although both mass spectra show similar fragmentation patterns, some fragments presented differences in relative intensities. In contrast to compound **IV**, compound **III** failed to give a molecular ion at m/z 505 (M) indicative of the dihydroxy-monooxo-pregnene structure. However, as depicted in Fig. 5, both present typical peaks at m/z 474 (M-31, base peak of compound **IV**), 415 (M-90), 400 [M-(15 + 90)], 384 [M-(31 + 90), base peak of compound **III**] and 294 [M-(31 + 2 × 90)]. Furthermore, the structurally informative ions can be observed at m/z 405 (M-100), 201, 170, 129 and 100

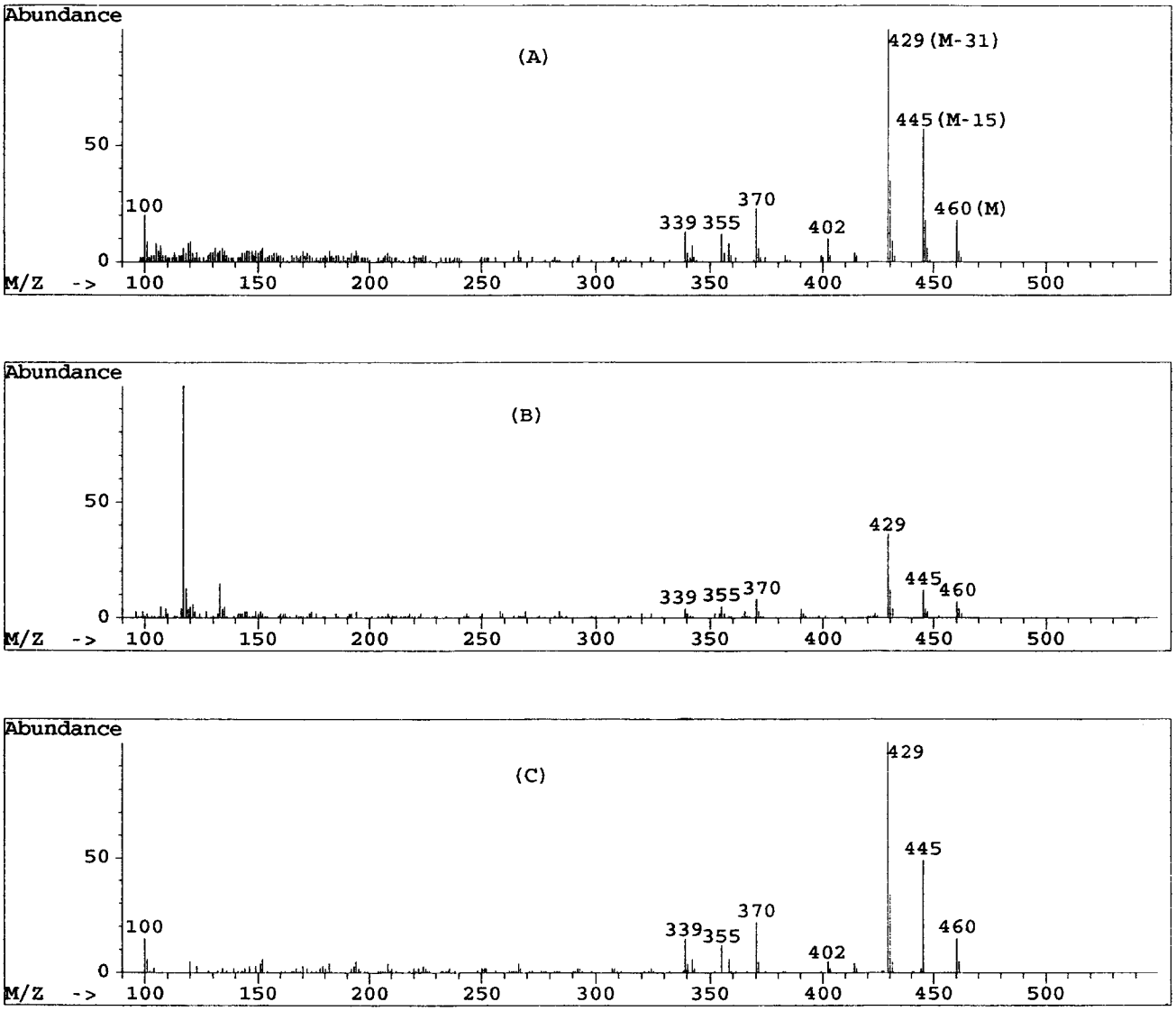

Fig. 2. Mass spectra of the MO-TMS derivatives of reference 2α-hydroxy-4-pregnene-3,20-dione (A), compounds I (B) and II (C). The peaks observed at m/z 117 and 133 (diagram B) are probably due to contaminating compound(s).

indicating a 15-trimethylsilyloxy-20-methoxyimino-structure with an otherwise unsubstituted D-ring and an acetyl side-chain [21–24]. This structure is also supported by the presence in the TMS ethers mass spectra (Fig. 6) of the peaks at *m/z* 129, 157 and 172. The last-mentioned peak is typical of the TMS ethers of 15- and 16-trimethylsilyloxy-20-oxo-steroids with an otherwise unsubstituted D-ring and an acetyl side-chain [25]. However, accompanied only by the peaks at *m/z* 129 and 157, it is strongly indicative of the 15-hydroxylated sterpids [13, 14]. In the mass spectra presented in Fig. 6, the small peaks indicated at *m/z* 476 are compatible with the molecular ion of bis-(trimethylsilyloxy)-monooxo-pregnene steroids. Fragments recorded at *m/z* 461 (M-15), 386 (M-90), 371 [M-(15 + 90)], 347 (M-129), 296 [M-(2 × 90)] and 257 [M-(129 + 90)] were in total agreement with the proposed structure. The fragment at *m/z* 129 and associated fragments, i.e. *m/z* (M-129) are often observed in steroids with a 3β-trimethylsilyloxy-5-ene-structure [25]. Previous results showed that the TMS ether mass spectrum of 3β,15β-dihydroxy-5-pregnen-20-one had given a base peak at *m/z* [M-(15 + 2 × 90)] [26]. In this work, the TMS ethers mass spectra of compounds **III** and **IV** also show the base peaks at *m/z* 281. Consequently, it seems reasonable to assume that these would appear to be the 3β,15-dihydroxy-5-pregnen-20-one isomers. Table 1 summarizes

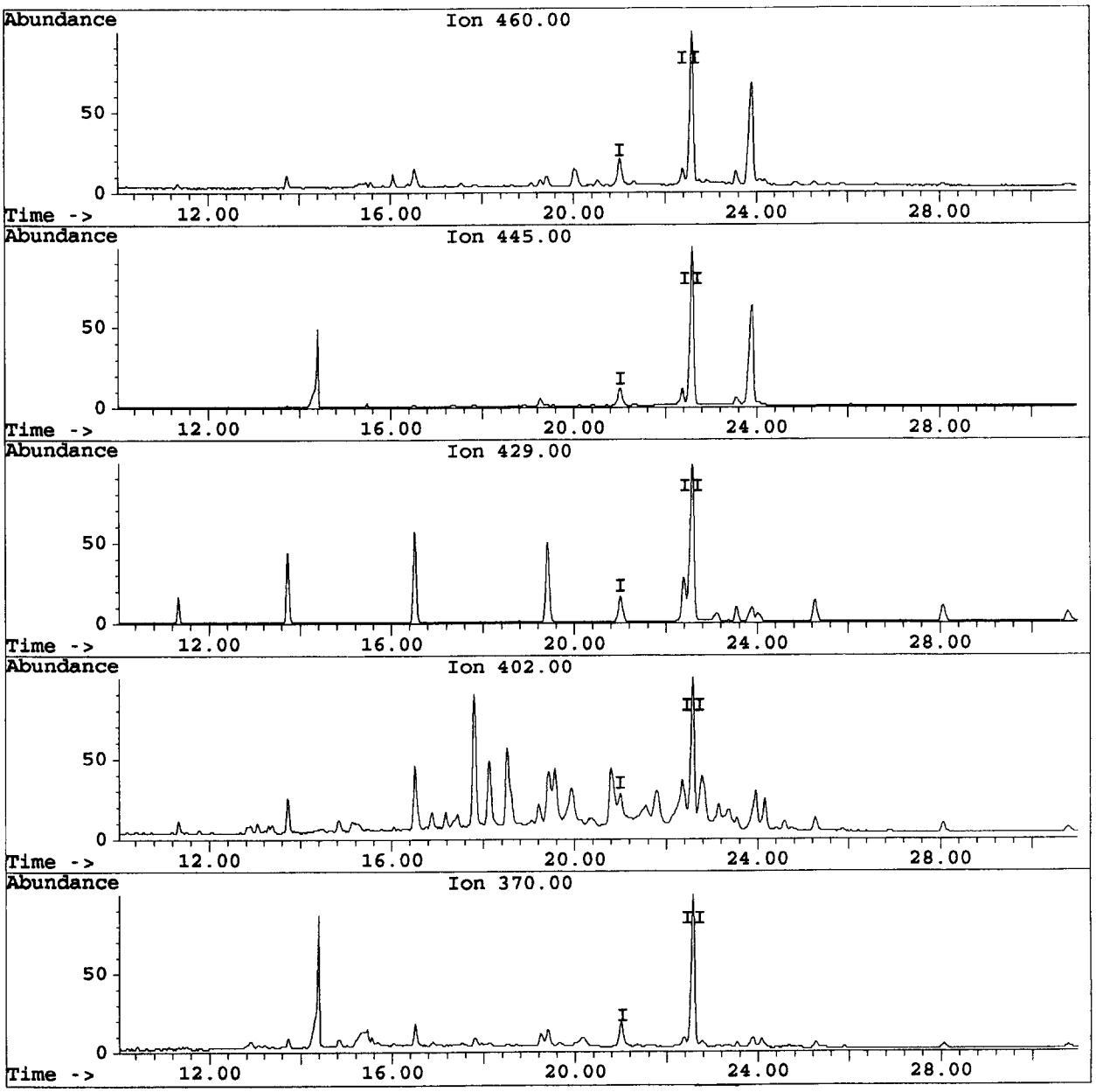

Fig. 3—*Caption overleaf.*

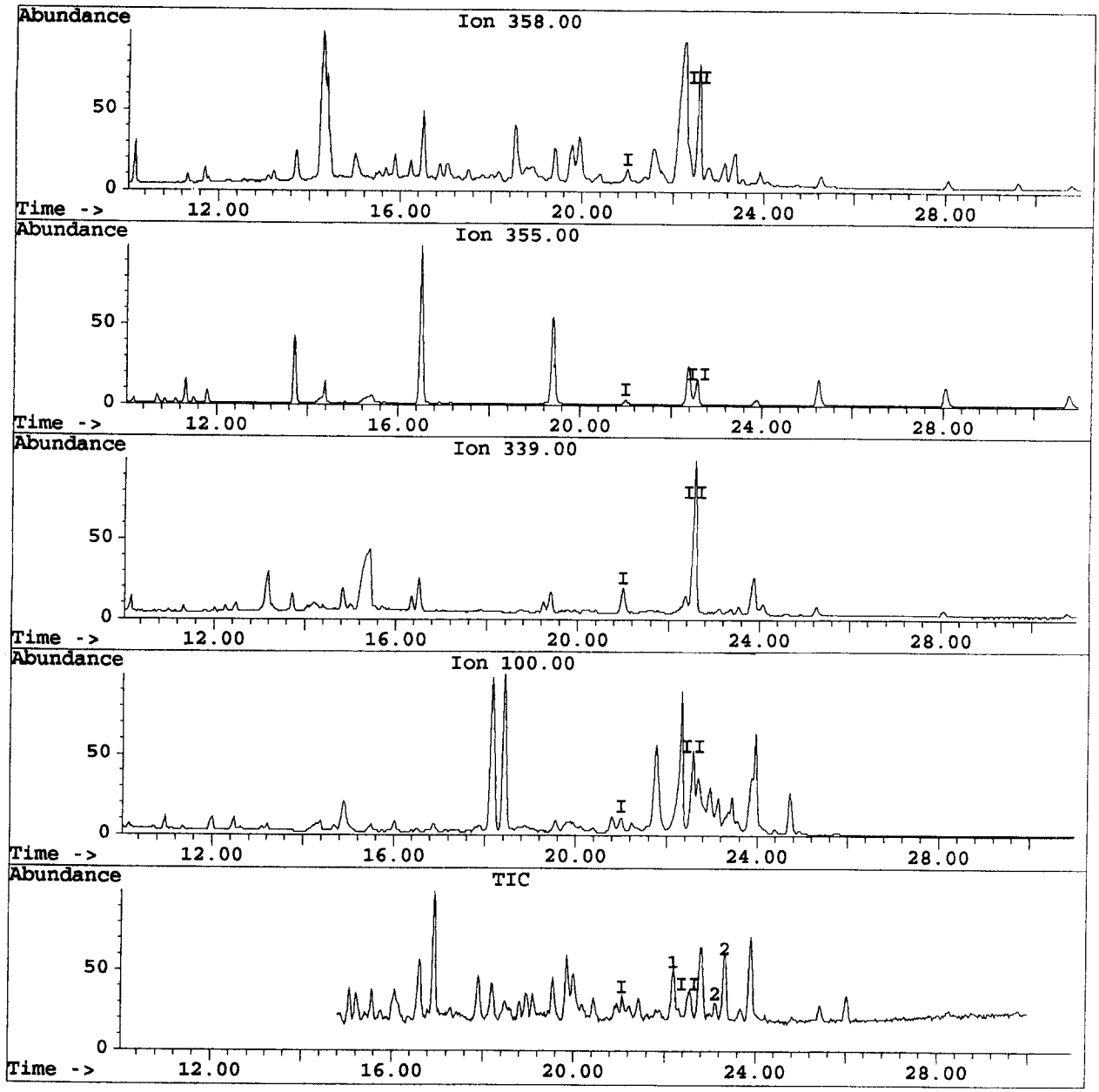

Fig. 3. SIM chromatograms of the ions representatives of the MO-TMS derivatives mass spectra of 2-hydroxy-4-pregnene-3,20-dione isomers. Indicated peaks correspond to compounds I and II characterized as 2β-hydroxy-4-pregnene-3,20-dione and 2α-hydroxy-4-pregnene-3,20-dione, respectively. Numbered peaks 1 and 2 were tentatively characterized as 16α-hydroxy-5β-pregnan-3,20-dione (MU, 28.93) and 16α-hydroxy-5α-pregnan-3,20-dione (MU, 29.43–29.53; probably *syn*/*anti*-isomers).

the chromatographic data obtained as a result of the GLC-MS analyses. Based on their chromatographic properties, their elution order was in agreement with previously published data of synthesized 3β,15β-dihydroxy-5-pregnen-20-one and 3β,15α-dihydroxy-5-pregnen-20-one as TMS ethers [26]. Additional and corroborating observations were obtained from SIM investigation. Figure 7 reproduces the SIM chromatograms of some ions informatives of compounds **III** and **IV** as TMS ethers. On the basis of all these observations, compounds **III** and **IV** were tentatively identified as 3β,15β-dihydroxy-5-pregnen-20-one and 3β,15α-dihydroxy-5-pregnene-20-one, respectively.

Other 15-hydroxylated steroids tentatively identified in this research work and which were also present in the Sephadex LH-20 fraction 14 were: 3β,15ξ,16ξ-trihydroxy-5-androsten-17-one, 5-androstene-3β,15α, 16α, 17β-tetrol, 3β,15β,17-trihydroxy-5-pregnen-20-one and 5-pregnene-3β,15ξ,17,20ξ-tetrol. Their identification was based on comparison of the chromatographic and mass spectrometric results obtained here with those found in the literature [5, 6, 27, 28].

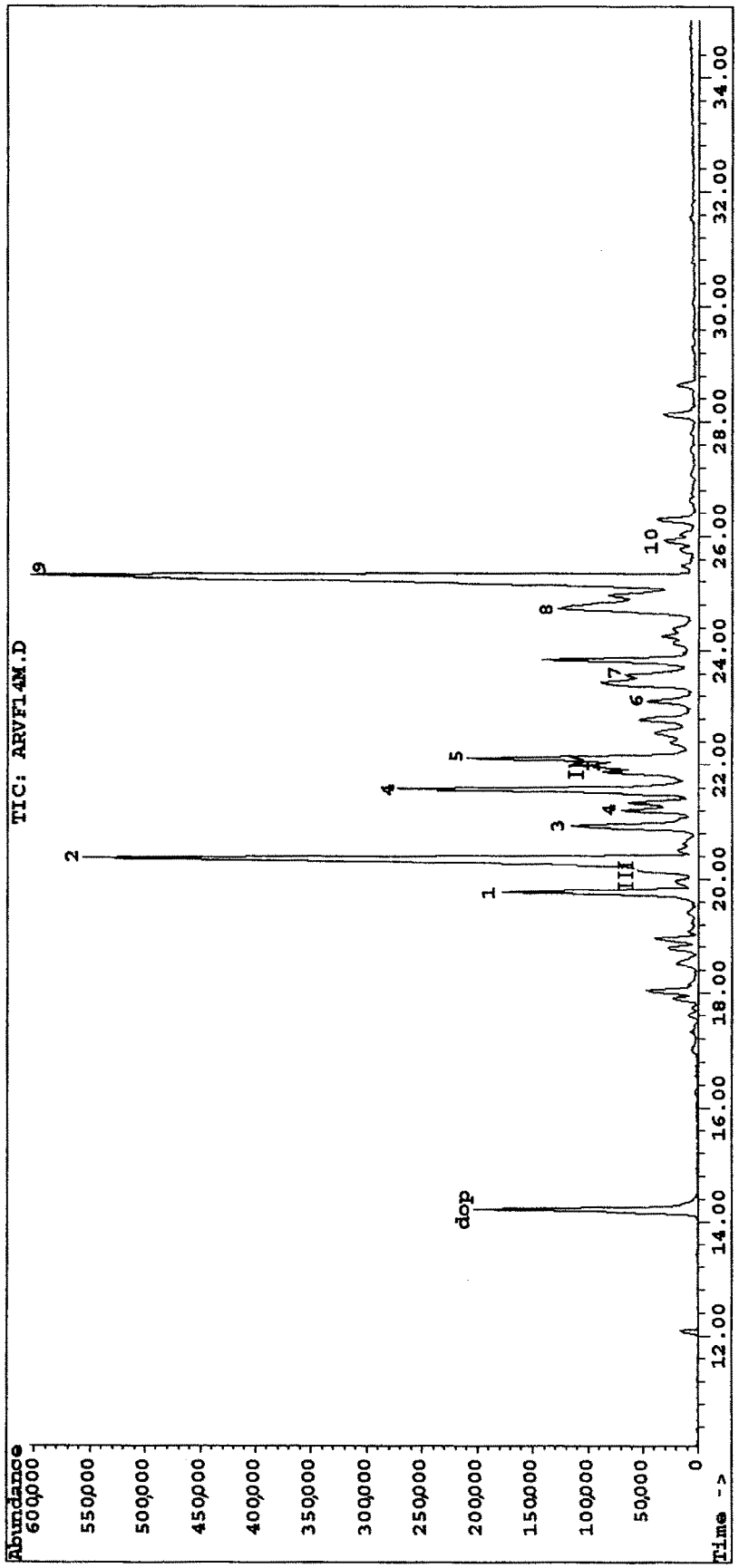

Fig. 4. TIC obtained during GLC-MS analysis of the MO-TMS derivatives of the steroids eluted in the Sephadex LH-20 fraction 14 from urine of one of the normal newborn human infants. For experimental conditions, see text. The numbered peaks were identified as: 1, 5-androstene-3β,16ξ,17ξ-triol (MU: 27.68); 2, 3β,15ξ,16ξ-trihydroxy-5-androsten-17-one (MU: 27.98); 3, 5-androstene-3β,16ξ,17ξ-triol* (MU: 28.29); 4, 3β,16ξ,18-trihydroxy-5-androsten-17-one (MU: 28.43–28.62; presumably *syn/anti*-isomers); 5, 3β,15β,17-trihydroxy-5-pregnen-20-one (MU: 28.90); 6, 5-androstene-3β,15ξ,17,20ξ-tetrol (MU, 29.42); 7, 5-pregnene-3β,15ξ,17,20ξ-tetrol (MU: 29.66; $TMSi_4$); 8, 5-androstene-3β,15α,16α,17β-tetrol (MU: 30.27); 9, 5-pregnene-3β,15ξ,17,20ξ-tetrol (MU: 30.56*; $TMSi_3$) and 10, 5ξ-pregnane-3ξ,6ξ,20ξ,21-tetrol (MU: 30.88). dop = dioctylphthalate. *Mixture of compounds.

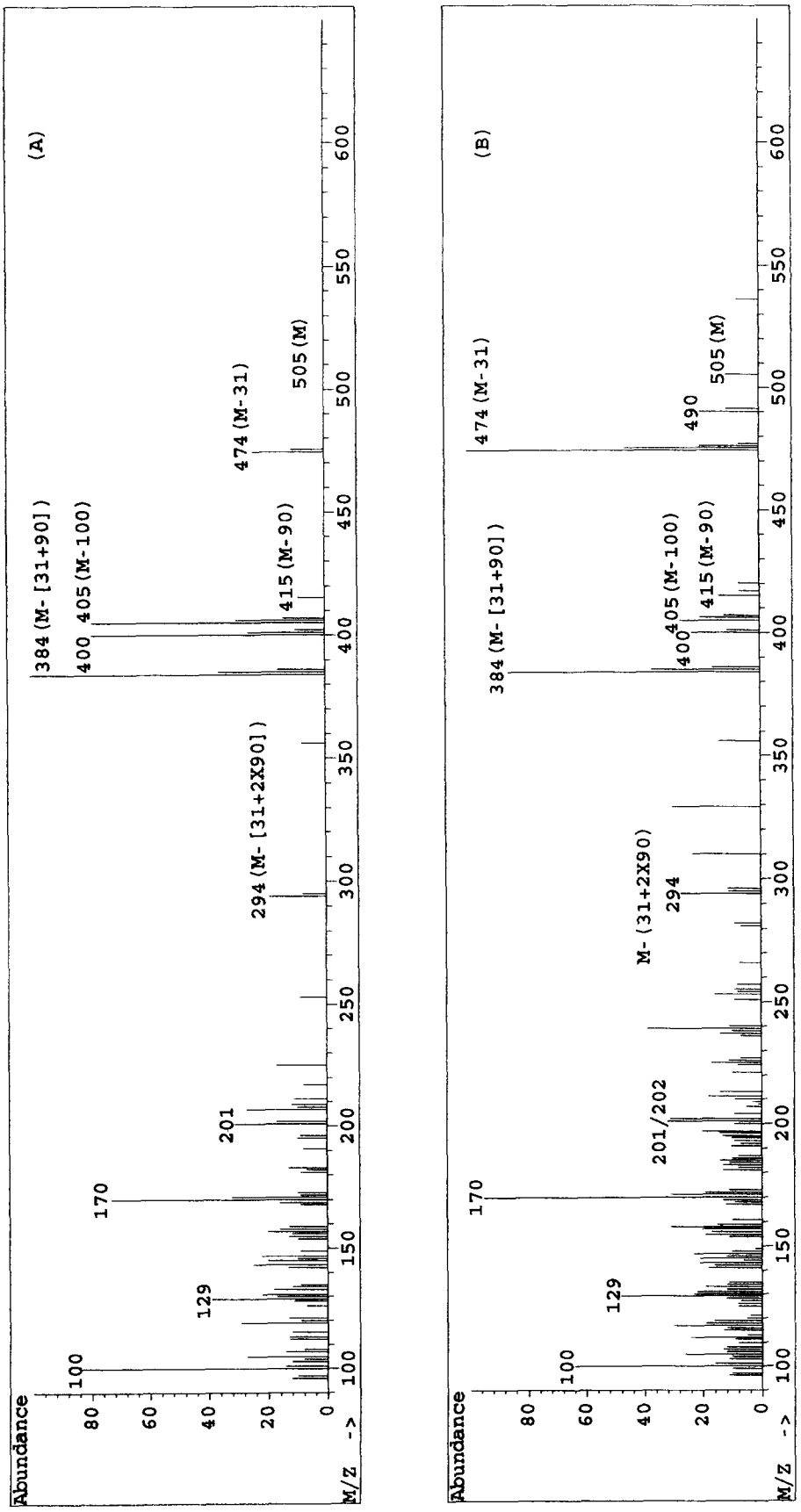

Fig. 5. Mass spectra of the MO-TMS derivatives of the urinary compounds tentatively identified as 3β,15β-dihydroxy-5-pregnen-20-one (A, compound III) and 3β,15α-dihydroxy-5-pregnen-20-one (B, compound IV).

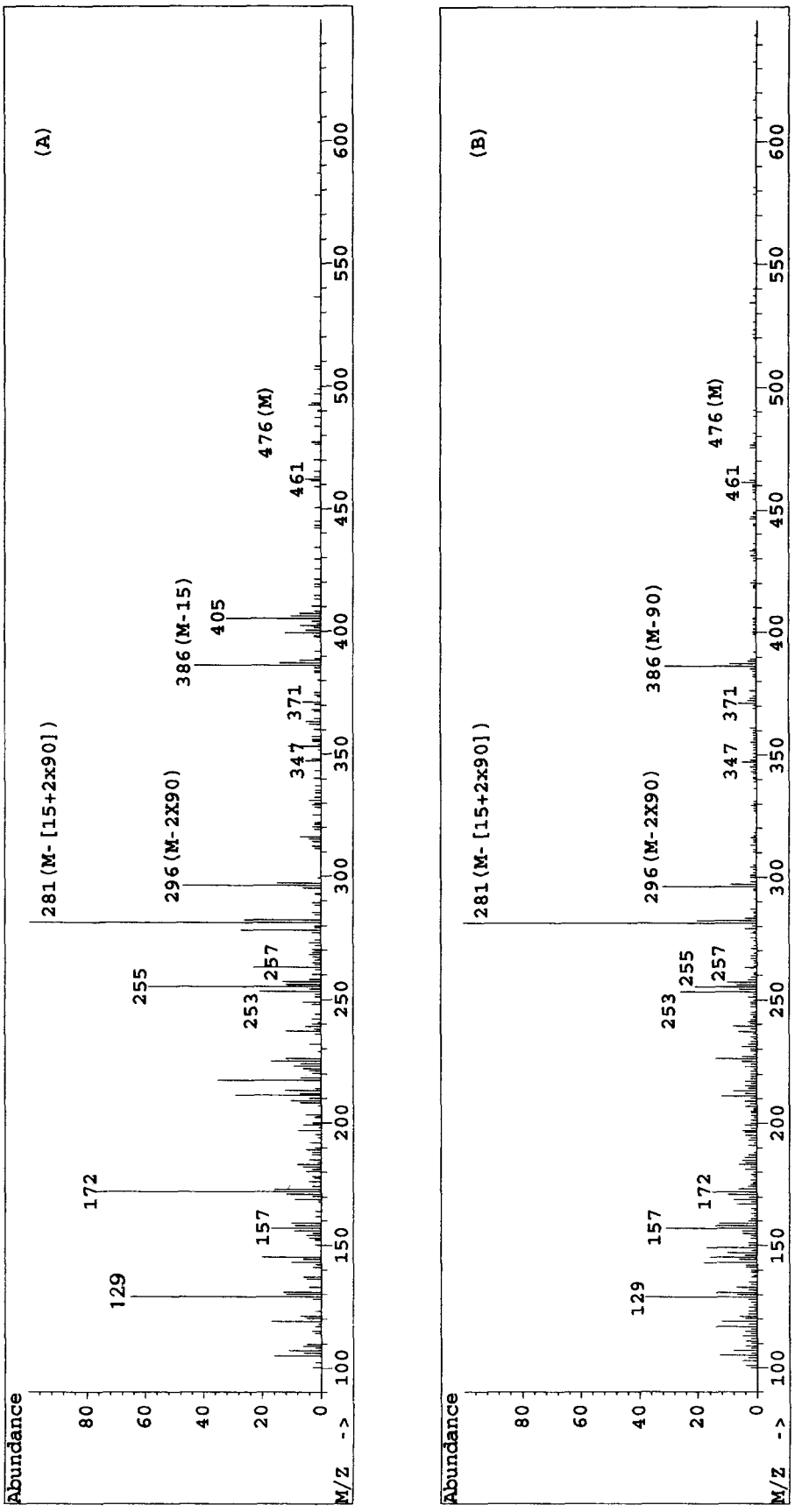

Fig. 6. Mass spectra of the TMS ethers of the urinary compounds tentatively identified as 3β,15β-dihydroxy-5-pregnen-20-one (A, compound III) and 3β,15α-dihydroxy-5-pregnen-20-one (B, compound IV). Some peaks observed such as the peak at *m/z* 217 (graph A) are presumably due to contaminating compound(s).

DISCUSSION

The identification of 2α-hydroxy-4-pregnene-3,20-dione and the characterization of 2β-hydroxy-4-pregnene-3,20-dione in this investigation shows for the first time the occurrence of such steroid metabolites in the urine of newborn human infants. 2α-Hydroxy-4-pregnene-3,20-dione has also been identified recently in human late-pregnancy urine [1]. In agreement with previous studies on progesterone metabolism in the human feto–placento–maternal complex [4, 10, 29], it was proposed that the fetal liver could be the principal site of 2α-hydroxylation of placental progesterone. At least indirectly, the present investigation seems to support this proposition. The case is probably the same for 2β-hydroxylation. The biological significance and physiological importance of 2-hydroxylation of progesterone in human neonates is still unknown, as in the case of pregnant women. The formation of 2α, and 2β,17β-dihydroxy-4-androsten-3-one after incubations of 17β-hydroxy-4-androsten-3-one with human fetal liver [16, 26, 29] and adrenal [29, 30] microsome-preparations, using a medium supplemented with a NADPH-regenerating system, has previously been demonstrated. It has also been demonstrated that the human fetal liver microsomes catalyze equally the 2α- and 2β-hydroxylation of 4-androstene-3,17-dione [19, 29]. Furthermore, the microsomal fraction from the liver of human adults also shows a 2-hydroxylase system active on some neutral steroids [31]. It appears that major microsomal *P*-450

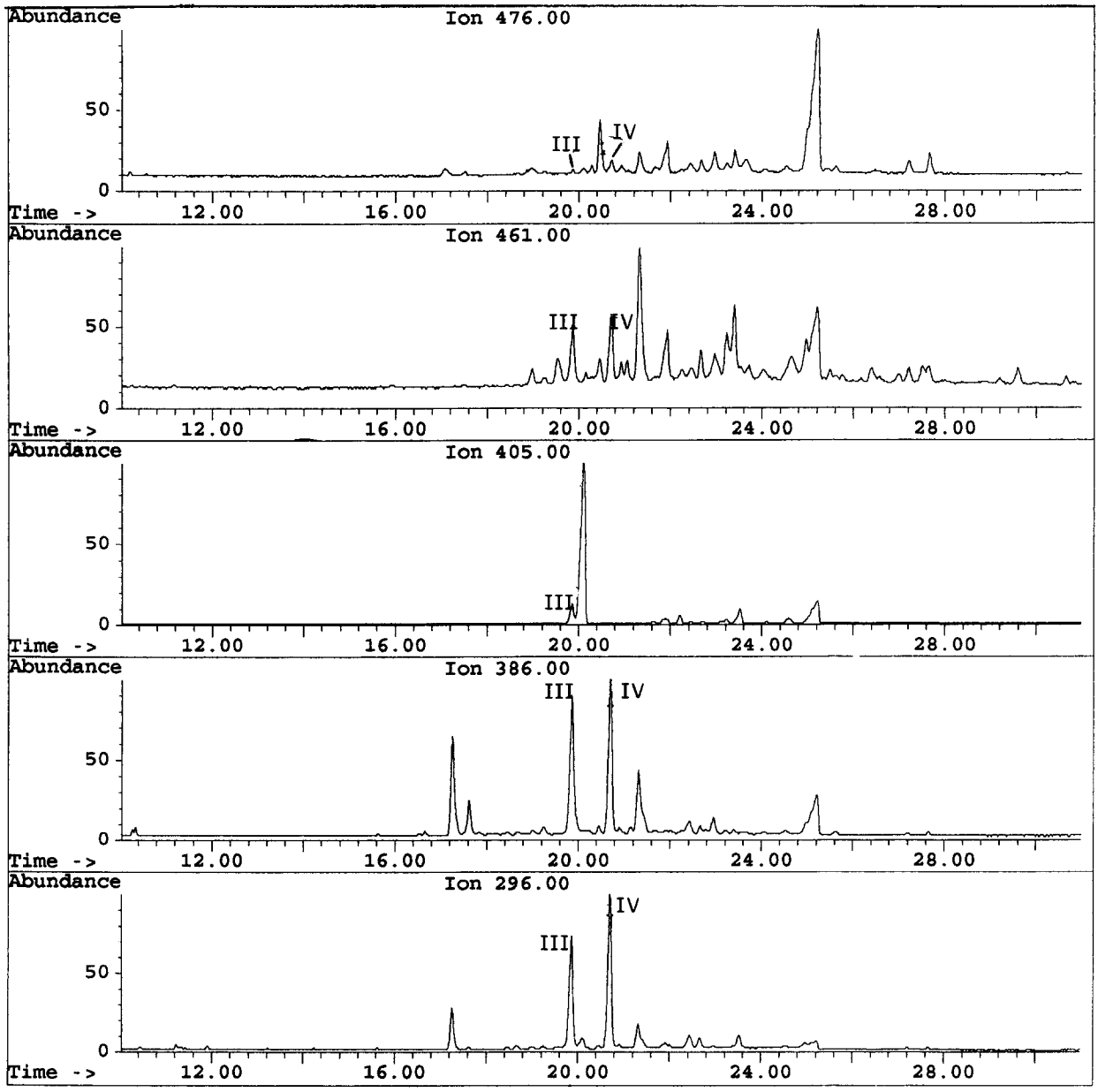

Fig. 7—*Caption on opposite page.*

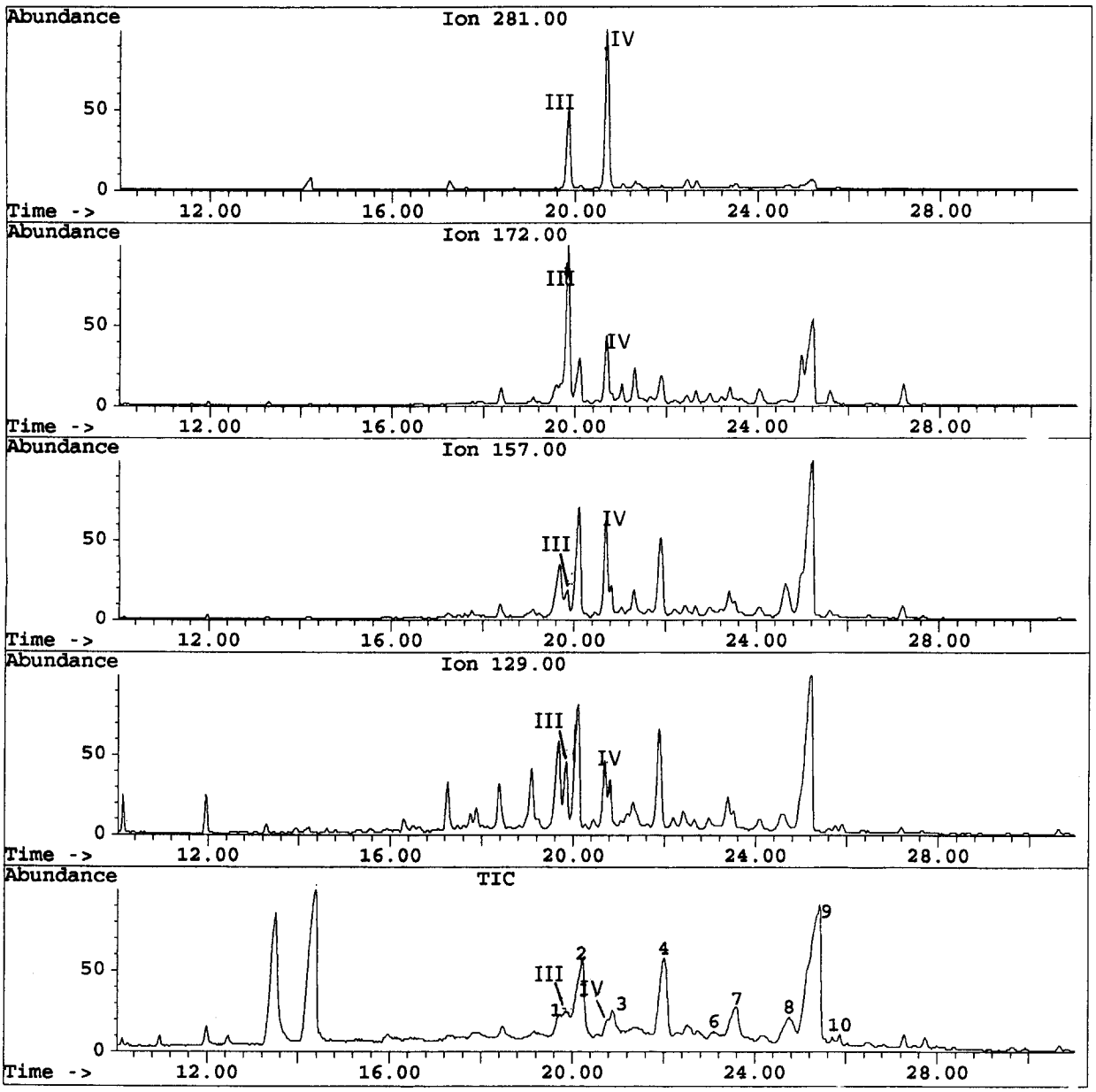

Fig. 7. GLC-MS analysis of the TMS ethers of the steroids eluted in Sephadex LH-20 fraction 14. This figure reproduces the SIM chromatograms of the fragments representative of the TMS ethers mass spectra of the compounds III and IV. Numbered compounds correspond to: 1, 5-androstene-3β,16ξ,17ξ-triol (MU: 27.66); 2, 3β,15ξ,16ξ-trihydroxy-5-androsten-17-one (MU: 27.91); 3, 5-androstene-3β,16ξ,17ξ-triol (MU: 27.95*); 4, 3β,16ξ,18-trihydroxy-5-androsten-17-one (MU: 28.84); peak chromatographic of 3β,15β,17-trihydroxy-5-pregnen-20-one cannot be localized as TMS ether; 6, 5-androstene-3β,16β,17β,18-tetrol (MU: 29.42); 7, 5-pregnene-3β,15ξ,17,20ξ-tetrol (MU: 29.66*; TMS_4); 8, 5-androstene-3β,15α,16α,17β-tetrol (MU: 30.27); 9, 5-pregnene-3β,15ξ,17,20ξ-tetrol (MU: 30.62*; TMS_3); 10, 5ξ-pregnane-3ξ,6ξ,20ξ,21-tetrol (MU: 30.86). dop = dioctylphthalate. *Mixture of compounds.

enzymes in human fetal and adult livers belong to the CYP3A gene subfamily [32]. In human pregnancy, it seems equally likely that the 2-hydroxylation of neutral steroids is not limited to maternal and fetal compartments. Recent *in vitro* experiments undertaken by Osawa *et al.* [33] have shown that the aromatase of human placental microsomes could catalyze the 2β-hydroxylation of 4-androstene-3,17-dione and 17β-hydroxy-4-androsten-3-one. As a result, therefore, it appears reasonable to assume that fetal and neonatal livers are the principal sites of 2-hydroxylation of progesterone. However, as for several steroid-metabolizing microsomal hydroxylase systems, the 2-hydroxylation of neutral steroids is not confined to human

tissues. Indeed, the biochemistry, molecular biology and physiology of the cytochrome *P*-450s involved in the hepatic biotransformation of the steroid hormones have been well demonstrated in the rat and other animals (see reviews [34–36]). For example, it has been established that the rat liver microsomes can hydroxylate the 3β-hydroxy-5-pregnen-20-one [37] and progesterone [38] to the corresponding 2β- and 2α-hydroxylated steroids. Furthermore, the 2α-hydroxylation of aldosterone by rat liver microsomes has also recently been demonstrated [39]. In male rat liver, the 3β-hydroxy-5α-pregnan-20-one sulfate was principally converted to the 2α-hydroxylated metabolites, namely 2α,3β-hydroxy-5α-pregnan-20-one and 2α,3α-hydroxy-5α-pregnan-20-one [40]. The results of Ramirez *et al.* [15] have also shown that the newborn rat adrenal cells in primary culture catalyze the epimerization of 2α-hydroxy-4-pregnene-3,20-dione to 2β-isomer. These results may therefore suggest that it is highly probable that in the human, as is also the case in the rat, the 2-hydroxylation is a function of the liver. In the perinatal period this could be mainly a function of the fetal and neonatal livers, although maternal and placental compartments cannot be excluded. To our knowledge, these 2-oxygenated C_{21}-steroids have not been present before in human neonates. This investigation shows that their identification by GLC and GLC-MS was made possible as a result of simplification and purification of urinary extracts, as far as we are concerned, by chromatography on Sephadex LH-20 column. Such chromatographic steps may prove imperative in the identification of other minor urinary steroid metabolites.

In this work, evidence has also been obtained to support the presence of 3β,15β-dihydroxy-5-pregnen-20-one and 3β,15α-dihydroxy-5-pregnen-20-one in neonatal urine. Of these two metabolites, the 3β,15α-dihydroxy-5-pregnen-20-one has already been identified in human late-pregnancy urine [11]. It was suggested then, that this steroid, could be a precursor of 15α-hydroxy-4-pregnene-3,20-dione and 3β,15α-dihydroxy-5-androsten-17-one both of which are known to be normal metabolites of pregnancy urine [8, 11, 41]. In addition to the above metabolites, the presence of other 15α-hydroxylated neutral steroids such as 15α-hydroxy-4-androstene-3,17-dione, 15α,17β-dihydroxy-4-androsten-3-one [42] and some 3,15-dihydroxypregnan-20-one isomers [22, 41] has been described. It is generally accepted that the 15α-hydroxylation is predominantly a function of the fetus. In normal pregnancy, it appears that at least partly the 15α-hydroxy-4-pregnene,3,20-dione could be derived from placental metabolism of fetal 3β,15α-dihydroxy-5-pregnen-20-one. Because of its 3β-hydroxysteroid deshydrogenase/5-ene-4-ene isomerase deficiency, *in utero* this biotransformation would be very limited or even absent in the fetal compartment. 3β,15α-dihydroxy-5-pregnen-20-one also could be converted to 3β,15α-dihydroxy-5-androsten-17-one by fetal adrenal *P*-450c17α *via* possibly 3β,15α,17-trihydroxy-5-pregnen-20-one. 3β,15β-dihydroxy-5β-pregnan-20-one and 3β,15β-dihydroxy-5α-pregnan-20-one have been identified in pregnancy urine by Eriksson and Gustafsson [23]. No 15α-hydroxylated neutral C_{21} steroids were found by these authors. As a result, it was then suggested that in pregnancy the 15β-hydroxylation is more important a reaction than the 15α-hydroxylation. However, these data might also reflect an extensive peripheral metabolism of 15α-hydroxylated metabolites [11]. Considering the distribution of steroid-metabolizing enzymes in the feto–placento–maternal system [4, 10], it is quite apparent that 15β-hydroxylase activity is mainly a fetal function. The *in vitro* incubation experiments, using human fetal liver microsomes, showed that 3β-hydroxy-5-pregnen-20-one sulfate was metabolized to 3β,15β-dihydroxy-5-pregnen-20-one and 3β,16α-dihydroxy-5-pregnen-20-one sulfates [26]. It was also established that 15β-hydroxylation requires a steroid sulfate-specific 15β-hydroxylase enzyme [26]. Urinary excretion during the neonatal period of 15β-hydroxylated metabolites such as 3β,15β,17-trihydroxy-5-pregnen-20-one and 3α,15β,17-trihydroxy-5β-pregnan-20-one was reported for the first time by Joannou [5]. Afterwards, it was suggested that 3α,15β,17-trihydroxy-5β-pregnan-20-one was considered as a good marker for the most common form of congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. These findings were recently supported by Kraan *et al.* [7], who not only showed the presence of 3α,15β,17-trihydroxy-5β-pregnan-20-one but also that of 3α,15β,17-trihydroxy-5α-pregnan-20-one, 5α- and 5β-pregnane-3α,15β,17,20α-tetrol. These latter compounds are known to be metabolites of 17-hydroxy-4-pregnene-3,20-dione [5–7]. The 5-pregnene-3β,15β,17,20α-tetrol was identified for the first time by Jänne and Vihko [22] in human late-pregnancy urine. As the 3β,15β,17-trihydroxy-5-pregnen-20-one, the 5-pregnene-3β,15β,17,20α-tetrol had previously been identfied in amniotic fluid [27], in umbilical cord blood [10], in the urine of normal [5] and pathological (CAH) newborn infants [6]. Probably, in human pregnancy and neonatal periods the 3β,15β-dihydroxy-5-pregnen-20-one might be metabolized to Jänne and Vihko's steroid *via* 3β,15β,17-trihydroxy-5-pregnen-20-one. Then, it also seems reasonable to suggest that 3β,15β-dihydroxy-5-pregnen-20-one could be one of the precursors of 3β,15β,16α-trihydroxy-5-androsten-17-one and 15β-hydroxyestriol. The latter two steroids have been shown to be normal urinary metabolites of human pregnancy and neonatal [3]. To our knowledge, neither 3β,15β-dihydroxy-5-pregnen-20-one nor 3β,15α-dihydroxy-5-pregnen-20-one have previously been reported in newborn human infant urine. However a definitive identification awaits comparison with the authentic steroids.

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