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DIURNAL VARIATIONS IN EXCRETION OF CORTICOSTEROID METABOLITES IN BILE FROM MALE AND FEMALE RATS

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SUMMARY

The excretion of corticosteroids in bile has been studied in female and male rats. The total daily excretion of corticosteroids in female rats was 738 ± 169 μg (3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one monosulphate: 300 ± 16 μg; 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one disulphate: 158 ± 51 μg; 3α,11β,21-trihydroxy-5α-pregnan-20-one disulphate: 146 ± 49 μg; and 3β,11β,21-trihydroxy-5α-pregnan-20-one disulphate: 134 ± 38 μg) and in male rats the corresponding figure was 773 ± 97 μg (3β,11β,21-trihydroxy-5α-pregnan-20-one disulphate: 682 ± 72 μg; and 5α-pregnane-3β,11β,20β,21-tetrol: 91 ± 25 μg).

The excretion of corticosteroids was cyclic in both female and male rats with the maximum excretion between 16·00 and 22·00 h and the minimum excretion between 04·00 and 10·00 h. The daily rhythm of corticosteroid excretion was disturbed for at least two days by operative manipulations in connection with bile duct cannulation, by intraperitoneal administration of saline and by intramuscular administration of dimethylsulphoxide.

INTRODUCTION

THE EXISTENCE of a circadian rhythm in corticosterone blood levels in the rat is well documented [1, 2]. However, it is not known how these diurnal variations are reflected in the excretion of corticosterone metabolites. The bile-fistula rat is a suitable experimental animal for studies on steroid hormone excretion in rats [e.g. 3, 4] and it was considered of interest to investigate in detail the intradaily variations of corticosteroid hormone excretion in bile from these rats and how the excretion is influenced by common experimental manipulations with the animals.

EXPERIMENTAL

Reference steroids

The sources of reference compounds have been given in previous publications [3, 4].

Conditions of animal experiments

The common bile duct was cannulated in Sprague–Dawley rats weighing about 200 g. Bile was collected every 6 h from 10·00 h (24 h after operation) for, usually, 48 h. The fistulated rats were given saline and were fed a pellet diet *ad libitum*. Two female and three male fistulated rats received no treatment whereas one female and one male rat were injected intraperitoneally at 10·00 h with about 0·5 μCi of [4 − ^{14}C]-corticosterone (S.A. 56·7 mCi mmol) in 0·5 ml of 1 % (w/v) bovine serum albumin in saline and another female and male rat were injected intramuscularly at the same hour with 0·5 ml. of dimethylsulphoxide (DMSO).

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Finally, one female rat was injected at 16·00 h with 15 I.U. of ACTH (kindly given by Organon, Holland) in 0·5 ml of saline after an initial bile collection period of 6 h. Bile was collected for 24 h. Five drops of chloroform were added to the bile samples when collected and the samples were stored at − 15°C.

Extraction and hydrolysis of biliary steroids

The bile was extracted with 10 volumes of acetone/ethanol, 1 : 1 (v/v), for 24 h at 39°C [5]. The extract was filtered and the filter paper was rinsed with 50 ml of acetone/ethanol, 1 : 1. After evaporation to dryness, the extract was dissolved in 2 ml of methanol, containing 0·02 M NaCl. When the extract had dissolved completely, 2 ml of chloroform were added, the mixture was ultrasonicated (Ultrasonic Generator Type L 364, Mullard Equipment Ltd., England) and then centrifuged for 10 min. The supernatant was removed, the sediment was resuspended in 4 ml of chloroform/methanol, 1 : 1 (v/v), containing 0·01 M NaCl, and the sample was ultrasonicated. After centrifugation, the supernatant was removed and combined with the first supernatant. The extract was applied to a 15 g column of Sephadex LH-20 prepared in chloroform/methanol, 1 : 1 (v/v), containing 0·01 M NaCl, [6]. The column was eluted with the same solvent mixture. The free steroid plus steroid glucuronide fraction was collected between 0 and 100 ml and the steroid monosulphate fraction between 100 and 250 ml of eluant [3]. The steroid disulphate fraction was eluted with 250 ml methanol.

The free steroid plus steroid glucuronide fraction was partitioned between 100 ml of ethyl acetate and 100 ml of 8·4 % (w/v) sodium bicarbonate in water. The ethyl acetate phase was washed with distilled water until neutral and was evaporated to dryness (free steroid fraction). The sodium bicarbonate phase was made neutral with 1 M HCl and was passed through a 10 g column of Amberlite XAD-2 [7]. The column was then washed with 50 ml of distilled water and the steroids were eluted with 100 ml of ethanol. The ethanol eluate was evaporated to dryness (steroid glucuronide fraction).

The fraction containing free steroids was trimethylsilylated and was analysed by gas chromatography–mass spectrometry (see below). It was found that the free steroid fraction contained no steroids, either in female or male rats.

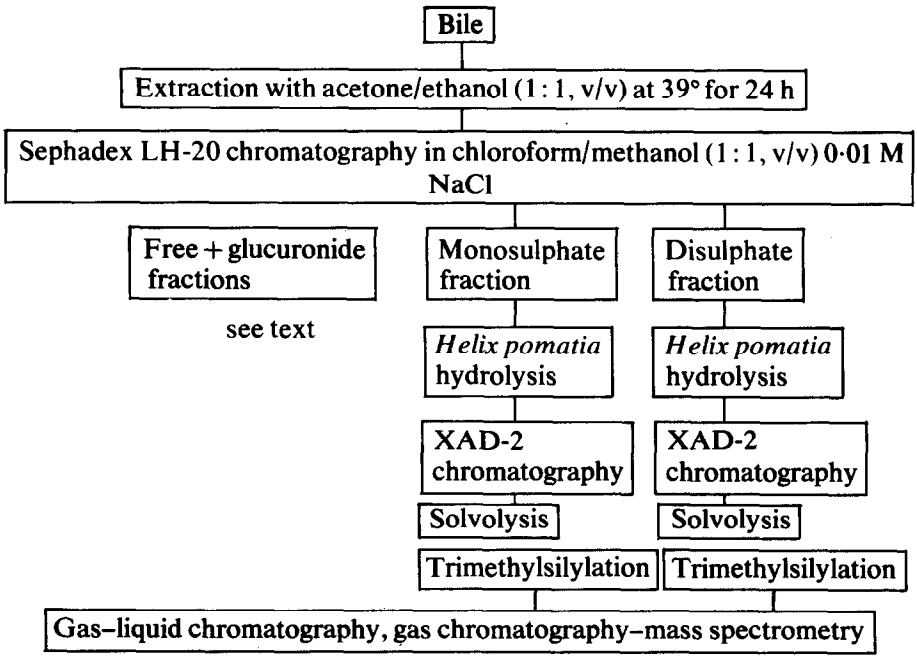
The steroid glucuronide fraction was dissolved in 14 ml of 0·5 M acetate buffer (pH 4·5) and 2 ml of Ketodase were added. The mixture was incubated at 37°C for 48 h, another 2 ml of Ketodase being added after 24 h. The mixture was then extracted with ethyl acetate. An aliquot of the extract was silylated and analysed by gas chromatography–mass spectrometry. The female control rats excreted the following steroid glucuronides, previously identified in bile from fistulated female rats [3]: 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5α,17α-pregnan-20-one, 3α-hydroxy-5α-pregnan-20-one, 5α-pregnane-3α,20β-diol and 3α,16α-dihydroxy-5α-pregnan-20-one. Ovariectomy resulted in disappearance of these steroids from bile (unpublished observations). No steroid glucuronides were seen in bile from male rats. Because of the quantitative unimportance of the steroid glucuronides, these steroids were not measured in the experiments described below.

The steriod mono- and disulphate fractions were evaporated to dryness and dissolved in 15 ml of redistilled water. Two ml of 0·5 M acetate buffer, pH 5·20 and 0·2 ml of digestive juice from *Helix pomatia* (Industrie Biologique Française, Genevilliers, France) were added and the mixture was incubated at 37°C for 48 h,

with addition of another 0·2 ml of digestive juice after 24 h of incubation. The incubation mixture was chromatographed on a 10 g column of Amberlite XAD-2. The column was washed with 50 ml of water and 2 ml of hexane and the steroids were eluted with 100 ml of ethanol. The ethanol extract was evaporated to dryness and was solvolysed. The liberated steroids were silylated by dissolving the sample in 0·5 ml of pyridine, and then adding 0·3 ml of hexamethyldisilazane and 5 drops of trimethylchlorosilane. This mixture was kept at 60°C for at least 12 h and was then evaporated to dryness and extracted with hexane. The silylated steroids were analysed by gas–liquid chromatography (see below).

The recoveries during the described extraction procedure were followed by measurements of radioactivity in a Packard Liquid Scintillation Counter (Model 4322) using Instagel[R] and internal quenching correction. Similar recoveries were obtained in male and female rats in the present investigation and in several unpublished experiments. During the first 6 h period after administration of radioactivity the rats excreted about 88–94 % of the injected radioactivity in bile. 80–90 % of the excreted radioactivity was recovered after extraction with acetone/ethanol (1 : 1, v/v). The following distribution of radioactivity was obtained after chromatography on Sephadex LH-20 (the figures given represent % of the radioactivity eluted from the Sephadex LH-20 column): free steroid fraction, 4–6 %; steroid monosulphate fraction, 17–27 %; and steroid disulphate fraction, 64–74 %. After combined hydrolysis with digestive juice from *Helix pomatia* and solvolysis and after trimethylsilylation, the total recoveries of mono- and disulphurylated radioactive metabolites were 68–88 and 77–88%, respectively.

The procedures employed for the extraction and purification of steroids in bile are summarized in the flow sheet below:



Analysis of biliary steroids

The gas–liquid chromatographic analyses were carried out on a Pye Gas

Chromatograph Model 64 (W.G. Pye & Co., Ltd., Cambridge, England) using 1·5% SE-30 as the stationary phase. Retention times (t_R) were determined relative to 5α-cholestane. The quantitations were based on comparisons between peak areas produced by steroids from bile with peak areas of known amounts of cholesteryl butyrate. The relative response factors (= (peak area of a μg of reference steroid/peak area of a μg of cholesteryl butyrate)) for reference 3α- and 3β,11β,21-trihydroxy-5α-pregnan-20-one were 0·92 ± 0·02 and 0·89 ± 0·02, respectively. Steroids in bile that were not available as reference compounds in amounts which could be weighed were quantitated assuming a response factor of 1·0. No corrections for losses during extraction and hydrolysis were made and therefore the figures of excreted compounds are minimum values. The lower limit of the method was 3μg/6 h.

All samples that were quantitated were also analysed by gas chromatography–mass spectrometry to identify the compounds and to control that the gas chromatographic peaks measured were homogeneous and consisted of steroids free from contaminating non-steroidal material. An LKB 9000 instrument was used for gas chromatography–mass spectrometry. The energy of the bombarding electrons was 22·5 eV and the ionizing current was 60 μA. Mass spectra were recorded on magnetic tape using the incremental mode of operation described by Hedfjäll *et al.* [8]. An IBM computer was used for processing the data [9]. The identifications of steroids were based on relative retention times and mass spectra.

RESULTS

Figure 1 (upper panel) shows the excretion of steroids in bile from an untreated female rat. It can be seen that all corticosteroids quantitated (mono- and disulphurylated 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one and disulphurylated 3α- and 3β,11β,21-trihydroxy-5α-pregnan-20-one) are excreted in largest amounts between 16·00 and 22·00 h and in smallest amounts between 04·00 and 10·00 h. A similar course in diurnal variation of corticosteroid excretion was also seen in the second untreated rat that was investigated in the same way. When mean values ($n = 4$) and standard deviations were calculated, the following figures were obtained for the daily steroid excretion ± one standard deviation: total corticosteroids: 738 ± 169 μg; monosulphurylated 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one: 300 ± 16 μg; disulphurylated 3α,11β,21-trihydroxy-5α-pregnan-20-one: 146 ± 49 μg; disulphurylated 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one: 158 ± 51 μg; and disulphurylated 3β,11β,21-trihydroxy-5α-pregnan-20-one: 134 ± 38 μg.

Figure 1 (middle panel) also shows the effects of intraperitoneal injection of 0·5 ml. of saline. It can be seen that a maximum corticosteroid excretion is already obtained between 10·00 and 16·00 h evidently as a result of the stress reaction evoked by the saline injection. The excretion minimum is still between 04·00 and 10·00 h and the second excretion maximum between 16·00 and 22·00 h. The total corticosteroid excretion during the first 24 h after saline administration is 920 μg and during the second 24 h 730 μg. Larger differences in total corticosteroid excretion between the first and second 24 h are seen after intramuscular administration of DMSO (Fig. 1, lower panel) when 859 and 490 μg, respectively, are excreted. The stress reaction induced by DMSO injection seems to lead to a depletion of the adrenal steroid content resulting in a much lower steroid excretion maximum than usual during the second day.

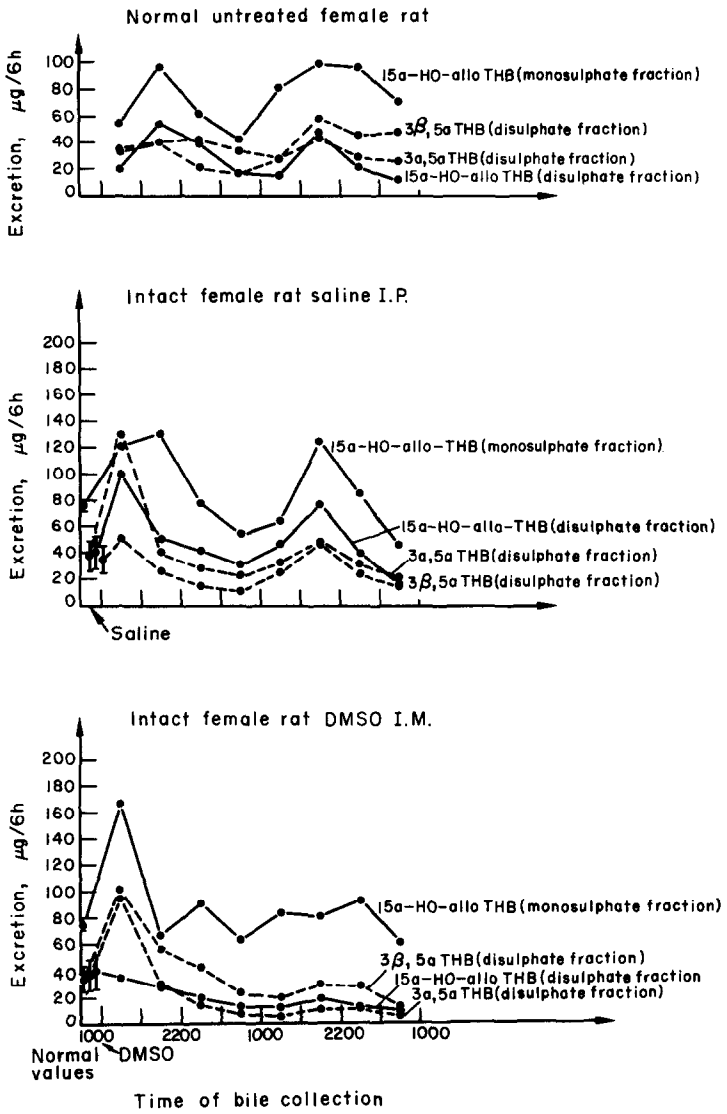


Fig. 1. Excretion of corticosteroids in bile from female rats during 48 h. Bile was collected every 6 h. Upper panel: untreated rat. Middle panel: rat given 0·5 ml of saline intraperitoneally at 10·00 h. Lower panel: rat given 0·5 ml of DMSO intramuscularly at 10·00 h. The normal values given to the left represent ½(± one S.D.) of the mean ($n = 4$) daily excretion of the steroid in question.

Abbreviations: 3α,5αTHB = 3α,11β,21-trihydroxy-5α-pregnan-20-one; 3β,5αTHB = 3β,11β,21-trihydroxy-5α-pregnan-20-one; 15α-HO-alloTHB = 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one.

In order to find out whether similar effects upon corticosteroid excretion to those observed after administration of saline and especially of DMSO could be produced by administration of ACTH, 15 I.U. of this hormone were injected intramuscularly in a female rat. The result is shown in Fig. 2. A sudden rise in corticosteroid excretion after injection of ACTH is followed by a period with low biliary corticosteroid excretion, the regular diurnal rhythm of steroid excre-

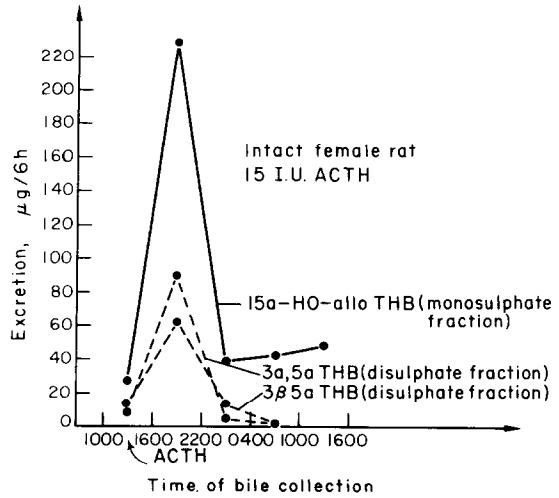


Fig. 2. Excretion of corticosteroids in bile from a female rat after intramuscular injection (at 16·00 h) of 15 I.U. of ACTH. Bile was collected every 6 h for 24 h. The normal values given to the left represent ¼(± one S.D.) of the mean ($n = 4$) daily excretion of the steroid in question.

Abbreviations: 3α,5αTHB = 3α,11β,21-trihydroxy-5α-pregnan-20-one; 3β,5αTHB = 3β,11β,21-trihydroxy-5α-pregnan-20-one; 15α-HO-allo THB = 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one.

tion is completely distorted. The results presented indicate that a single dose of ACTH effectuates a rapid depletion of the adrenal corticosteroid content but that no significant increase in corticosteroid biosynthesis ensues.

Figure 3 shows the biliary excretion in normal male rats. As can be seen from the upper panel there is a maximum of corticosteroid excretion between 16·00 and 22·00 h during the second 24 h collection period. During this period considerably larger amounts of corticosteroids are excreted (650 μg/24 h) than during the first collection period (290 μg/24 h). Only one excretion curve is shown from an untreated male rat but similar uneven excretion curves were obtained from two other untreated male rats. The following mean values and standard deviations were calculated from figures obtained during the second 24 h collection period from the three untreated male rats: total corticosteroids: 773 ± 97 μg; disulphurylated 3β,11β,21-trihydroxy-5α-pregnan-20-one: 682 ± 72 μg; and disulphurylated 5α-pregnane-3β,11β,20β,21-tetrol: 91 ± 25 μg. These values are similar to figures obtained in other experiments for daily corticosteroid excretion in bile from male rats[10] It is concluded that male rats require about 48 h after bile duct cannulation to resume a normal excretion pattern of corticosteroids in bile.

When saline was administered intraperitoneally (Fig. 3, middle panel), 540 and 690 μg, respectively, of total corticosteroids were excreted during the first and second 24 h periods. The corresponding figures after DMSO injection (Fig. 3, lower panel) were 550 and 430 μg, respectively. It can be seen that administration of DMSO brings about a more pronounced derangement of the circadian rhythm of corticosteroid excretion than administration of saline.

DISCUSSION

It has previously been demonstrated by several groups that the level of plasma

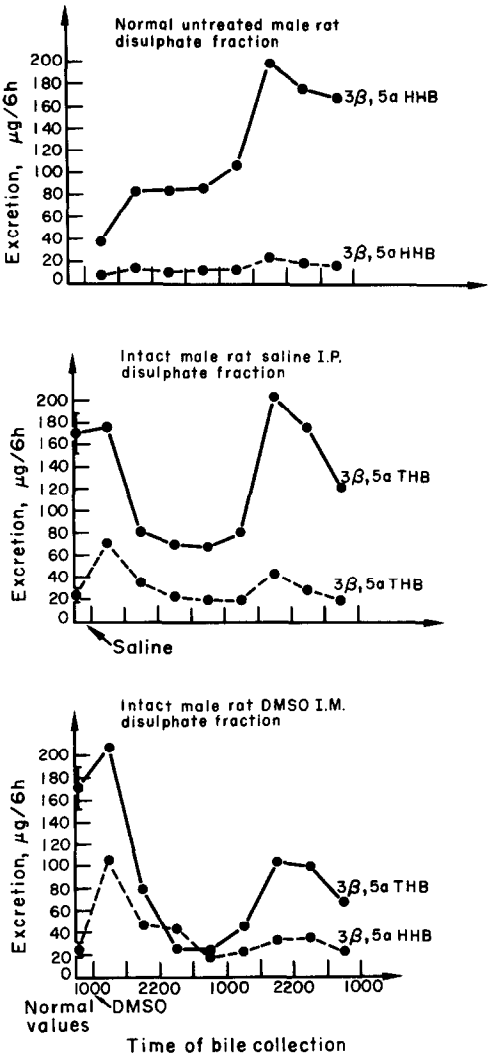


Fig. 3. Excretion of disulphurylated corticosteroids in bile from male rats. No measurable amounts of steroids were found in the steroid monosulphate fraction. Bile was collected every 6 h for 48 h. Upper panel: untreated rat. Middle panel: rat given 0·5 ml of saline intraperitoneally at 10·00 h. Lower panel: rat given 0·5 ml of DMSO intramuscularly at 10·00 h. The normal values given to the left represent ½(± one S.D.) of the mean ($n = 4$) daily excretion of the steroid in question.
Abbreviations: 3β,5αTHB = 3β,11β,21-trihydroxy-5α-pregnan-20-one; 3β,5αHHB = 5α-pregnane-3β,11β,20β,21-tetrol.

corticosterone in rats undergoes a diurnal variation with the peak concentration occurring at about the end of the light period and the beginning of the dark period [1, 2]. Since the biological half-life of plasma corticosterone in the rat is about 10–20 min [11, 12] and since intraperitoneally administered [4 − ^{14}C]-corticosterone is practically completely excreted in the bile of a bile-fistula rat within 2·5 h [4] it is not surprising to find that the excretion of biliary corticosteroid metabolites is also subjected to diurnal variation. The times found for maximum (between 16·00 and 22·00 h) and minimum (between 04·00 and 10·00 h)

excretion agree well with the times found by David-Nelson and Brodish for maximum (18·00 h) and minimum (09·00 h) plasma corticosterone levels [1].

Rhythmic changes in hepatic metabolism of a circadian character have been reported for certain steroid hydroxylases [13, 14]. In the present experiment, however, no diurnal variations in hydroxysteroid oxidoreductase or 15α-hydroxylase activities were evident *in vivo*, since the ratio between the different corticosterone metabolites in bile is about the same throughout the whole 24 h cycle. Furthermore, this ratio was not changed during the increased adrenal corticosterone output after an injection of ACTH, indicating that the hepatic corticosteroid metabolizing enzymes normally work considerably below substrate saturation.

A common manipulation with the rats such as intraperitoneal administration of saline is shown to have moderate but definite effects on the biliary corticosteroid excretion as long as about 36 h after saline injection. The effects of intramuscular adminstration of DMSO are more profound and are evident throughout the whole investigated 48 h period after injection. The saline and especially the DMSO administration thus seem to induce a stress reaction with a probably ACTH-mediated increase in adrenal steroid output. The results may indicate that the effect produced by ACTH is rather a depletion of adrenal corticosteroid depots than an accelerated corticosterone biosynthesis.

The corticosteroid excretion curves obtained for untreated male rats show that the effects of stress in connection with bile duct cannulation influences corticosteroid excretion also during the second postoperative day. This suggests that bile from a male bile-fistula rat should not normally be collected until 48 h after operation when the animals have resumed a normal corticosterone excretion pattern.

The results presented demonstrate that the bile-fistula rat is a very suitable experimental animal for studies on corticosteroid excretion. Reproducible figures of excreted amounts of steroids are obtained showing moderate individual variations among different rats. Circadian and artificially induced fluctuations in corticosteroid secretion can conveniently be followed by short-interval collections and analysis of biliary samples.

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