Influence of spironolactone on neonatal screening for congenital adrenal hyperplasia

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Abstract

Aim—To determine if the diuretic spironolactone cross reacts with 17 α -hydroxyprogesterone (17OHP) in an enzyme linked immunosorbent assay (ELISA) kit used for the mass screening of congenital adrenal hyperplasia.

Methods—Concentrations of 17OHP on a blood filter paper disc were measured using an ELISA kit (kit C-7: ENZAPLATE N-17 α -OHP-7; Chiron, Tokyo, Japan). The cross reactivity of spironolactone and its metabolites with 17OHP was determined. The concentrations of spironolactone and its metabolites in blood were measured using HPLC (high performance liquid chromatography).

Results—Spironolactone cross reacted with 17OHP using kit C-7 (0.01%), by increasing 17OHP concentration in a dose dependent manner. The blood concentration of spironolactone and its metabolites was nearly 900 ng/ml, high enough to show an additive effect on the 17OHP concentration. About 12% of the false positive cases screened using the kit were due to the administration of spironolactone.

Conclusions—Spironolactone interferes with 17OHP concentrations, leading to false positive test results for CAH.

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Keywords: screening; congenital adrenal hyperplasia; 17 α -hydroxyprogesterone; spironolactone

Neonates are screened for congenital adrenal hyperplasia using an enzyme linked immunosorbent assay (ELISA) or a radioimmunoassay for 17 α -hydroxyprogesterone (17OHP) on a blood filter paper disc.^{1–4}

In Japan most screening samples are obtained between days 4 and 7 of life, and 17OHP on blood filter paper is measured using an ELISA at an officially designated local laboratory.^{5–7} At present, any of the four commercially available kits—kit C-7, kit C-3, kit E, or kit K—are used in Japan. We use kit C-7. The blood specimen is assayed initially without extraction by organic solvent (direct assay); when an initial result is above the cutoff level, the same sample is usually rechecked using the same procedure, and simultaneously extracted by diethyl ether and measured again (extraction assay) using the ELISA. This is because diethyl ether extraction rarely identifies hydrophilic steroids such as 17 α -hydroxypregnenolone-3-sulphate^{8–10} and dehydroepiandrosterone sulphate,^{11–13} which are released in the blood of premature babies

and/or stressed babies, and so this procedure reduces the frequency of false positive test results.^{8 11 12 14}

Like most Japanese laboratories, we have adopted two concomitant cutoff levels: the percentile and the absolute value of 17OHP. We use the 95th percentile for direct assays. The cutoff level for the extraction assay is set at 3.5 ng/ml (10.6 nmol/l) blood. When the 17OHP concentration in the first sample measured by direct assay shows remarkably high values (more than 30 ng/ml (90.8 nmol/l) for the infant weighing more than 2000 g), the extraction assay is omitted, and the baby is promptly referred to the paediatric specialist at the hospital. Cases with high 17OHP values by extraction assay are also referred without delay, while those with slightly raised concentrations are requested to return for a second sampling.^{5–7}

Many of the repeat specimens were from infants with congenital heart disease who were receiving diuretics and digoxin. We suspected that spironolactone, a diuretic, and/or digoxin, both of which have a steroid structure, cross reacted with 17OHP in the 17OHP ELISA kit, producing a false positive result.

Methods

Dried blood spots on filter paper, taken at days 4 to 7 of life, for neonatal screening for inborn errors of metabolism, including congenital hypothyroidism and CAH, were collected from the Hokkaido prefecture excluding Sapporo city. The study was carried out in accordance with the Helsinki declaration, with informed consent from the parents.

The concentration of 17OHP on filter paper blood spots was measured using an ELISA kit. For analysis, a 3 mm diameter disc was punched out from the inner circles of each blood spot area. Standards and controls provided in the kit consisted of dried blood spots with 17OHP of known concentrations. For the assay, the manufacturer's instructions were followed at all stages of the procedure. Namely, each filter disc was directly put into microtitration wells coated with goat anti-rabbit IgG antibody (direct assay method), and the reaction was started by the addition of 50 μ l horseradish peroxidase labelled 17OHP (17OHP-HRP conjugate) and 100 μ l rabbit antiserum specific for 17OHP. The microtitre plate was covered with a plate seal and incubated for 18 hours at 25°C. The discs were then removed and the plate was washed three times with the detergent provided in the kit. After removing the washing solution 150 μ l of colour coupler solution (hydrogen peroxide

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and *o*-phenylenediamine) were added into each well, and the plate was incubated at 25°C for 30 minutes in the dark. The reaction was stopped by the addition of 100 µl termination solution, and the absorbency was read at 492 nm using a microplate reader (MTP-120, Corona electric Co., Katsuta, Japan). 17OHP concentrations were calculated using a computer programmed with the manufacturer's calculating software.

The extraction assay method was performed in duplicate using diethyl ether as an organic solvent.^{1,2,8} One filter disc (3 mm in diameter) containing dried blood was transferred to a test tube (50 mm × 6 mm) containing deionised water (200 µl), shaken vigorously for 6 minutes using a multi-tube vortexer, and then kept still overnight at room temperature. The 17OHP released into the water was extracted with diethyl ether (1 ml) by mixing for 2 minutes and spinning at 3000 rpm for 5 minutes at 4°C. Separation of the organic phase was carried out by freezing the watery phase by means of a refrigerating mixture (dry ice and ethanol). The diethyl ether extract was transferred to another test tube, and the organic phase was completely evaporated to dryness by heating at 55°C. Seventy five microlitres of peroxidase labelled 17OHP were added to the resultant residue, 50 µl aliquots of which were subjected to the ELISA described above.

Compounds with a steroid structure, including spironolactone, digoxin, spironolactone metabolites, cortisol, dexamethasone, cortisone, prednisolone, and aldosterone dissolved in dimethylsulphoxide at 10 ng/ml were further diluted with methanol or water. Aliquots of steroid solutions at different concentrations were added in duplicate directly into the wells of the ELISA. The percentage cross reactivity was calculated at 50% displacement of the 17OHP-HRP conjugate. This experiment was performed with four commercially available 17OHP neonatal screening kits. For each assay, the respective manufacturer's instructions were followed. An additive effect of spironolactone to the 17OHP value in kit C-7 was tested for on filter discs (3 mm in diameter) coated with a constant amount of 17OHP, together with an increasing amount of spironolactone. Spironolactone, frusemide (also known as furosemide or Lasix), and digoxin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Potassium canrenoate (Soldactone) was purchased from Searle (Skokie, ILL, USA). 7 α -thiomethylspironolactone, canrenone, and 6 β -hydroxy-7 α -thiomethylspironolactone, which are the main metabolites of spironolactone,¹⁵ were kindly donated by Searle. Cortisol, dexamethasone, cortisone, prednisolone, and α -aldosterone were purchased from Sigma Chemical Co. (MO, USA).

A quantitative analysis of spironolactone and its metabolites in dried blood spots on filter paper was accomplished by modifying the previously described method.¹⁶⁻¹⁹ One filter disc (10 mm in diameter) containing dried blood was immersed in 1 ml of 0.05 M phosphate buffer, pH 8.0, and sonicated for 10 minutes. Then, 10 ng (30 µl) of 19-nortestosterone in

60% methanol was added to the solution as an internal standard, and the resultant mixture was applied to the Extrelut column 1 (Merck & Co., Inc., Rahway, NJ, USA). Steroids adsorbed to the column were then eluted with dichloromethane (twice with 5 ml), and the eluate was completely evaporated to dryness by a nitrogen stream. The resultant residue was dissolved with 60 µl of 60% methanol, 30 µl aliquots of which were analysed by a reversed phase HPLC on an ERC-ODS-1171 column (ERMA CR, INC. Tokyo, Japan).

Results

When filter paper discs coated with increasing amounts of spironolactone with or without a constant amount of 17OHP (3.4 ng/ml = 10.3 nmol/l) were measured for 17OHP by a direct assay using the ELISA kit C-7, 17OHP values rose according to the amount of spironolactone added (fig 1). The curves produced by spironolactone alone on the discs and by spironolactone with a constant amount of 17OHP, paralleled each other, indicating the additive effect of spironolactone. The same phenomenon was also observed in the extract assay (not shown). Results suggested that spironolactone cross reacts with antibodies to 17OHP used in kit C-7. Percentage cross reactivity was determined for spironolactone and its metabolites using several different ELISA kits.

As shown in table 1, the cross reactivity of spironolactone with 17OHP in kit C-7 was calculated to be 0.01% ($1.1 \times 10^{-2}\%$), and those of its metabolites at the level of 10^{-3} to $10^{-5}\%$. In other kits (E, K, C-3), the percentage cross reactivity of spironolactone with 17OHP was in the order of 10^{-4} to 10^{-6} —much lower concentrations than those obtained with kit C-7 (table 1). Cross reactivity of other steroids (cortisol, dexamethasone, cortisone, prednisolone, and aldosterone) in kit C-7 was at the level of 10^{-3} to $10^{-6}\%$ (data not shown), and no cross reactivity was found for digoxin and frusemide.

Figure 1 Additive effect of spironolactone plotted against the 17OHP value in kit C-7 (direct assay): closed circles indicate 17OHP values obtained from filter discs coated with increasing amounts of spironolactone and a constant amount of 17OHP (3.4 ng/ml or 10.3 nmol/l blood).

Table 1 Cross reactivity of spironolactone and its metabolites with 17OHP

Steroid	Kit C-7	Kit C-3	Kit E	Kit K
Spironolactone	1.1×10^{-2} (%)	1.6×10^{-6}	1.3×10^{-4}	1.7×10^{-4}
Canrenoate	1.2×10^{-5}	1.1×10^{-6}	1.9×10^{-7}	1.4×10^{-5}
Canrenone	3.0×10^{-4}	ND*	3.2×10^{-6}	ND
7 α -Thiomethylspironolactone	6.2×10^{-3}	ND	1.8×10^{-5}	ND
6 β -Hydroxy-7 α -thiomethylspironolactone	2.5×10^{-5}	ND	1.1×10^{-6}	ND

*Not done

CASE REPORTS

Case 1 (fig 2)

A boy born by normal vaginal delivery at 39 weeks and weighing 3308 g was discharged on day 5. Although screening at day 5 for CAH was normal, with a value of 3.7 ng/ml (11.2 nmol/l) 17OHP by direct assay, the screening result for galactosaemia was slightly positive. A second sample of blood for galactosaemia was obtained on day 13. Subsequent to discharge, the infant developed a cough and severe oedema due to congestive heart failure arising from a ventricular septal defect, and was treated with frusemide, spironolactone, and digoxin. Figure 2 shows that before treatment, the 17OHP concentration by direct assay in kit C-7 showed a slight increase, but that by extraction assay it was within normal limits. This initial increase in 17OHP was considered to be due to some hydrophilic steroid released by the stress of the congestive heart failure. However, two days after the administration of the drugs, the 17OHP concentration became very high in both direct and extraction assays. After the administration of diuretics, the patient progressed satisfactorily, and 17OHP concentration decreased gradually. This decrease coincided with the decrease in spironolactone per weight per day (mg/kg/day) (fig 2). From the same sample, 17OHP was also measured by kits E and C-3, by direct assay. In kit E, an abnormally high 17OHP value was noted when congestive heart failure was at its peak, before the administration of diuretics. However, the values decreased as the condition improved with the administration of diuretics. This abnormal 17OHP peak coincided with the transient increase in body weight due to congestive heart failure during the course of overall weight loss (fig 2). The extraction assay using kit E, however, showed that every concentration was within normal limits. A similar result was obtained with kit C-3 (not shown). These results indicate that, in the direct assay, both kits E and C-3 pick up some hydrophilic steroids which are probably released into the blood as a result of severe stress.

Case 2 (fig 3)

A boy born at 39 weeks and weighing 3064 g had a complex coarctation of aorta. Spironolactone was administered soon after the birth, the dose increasing in proportion to his weight gain. In this case, 17OHP values measured by kit C-7 in both direct and extraction assays remained high during the administration of spironolactone but decreased to normal once the drug was stopped. Using kits E and C-3 (data not shown) in both direct and extraction assays, the time course changes in 17OHP concentrations paralleled those seen with kit

C-7, although every value remained within normal limits.

Case 3 (fig 4)

A boy born at 29 weeks of gestation and weighing 1310 g at birth received frusemide for oedema due to immaturity of the kidney. The 17OHP concentration showed a slight increase by direct assay and a normal value by extraction assay in kit C-7. After the baby began treatment with spironolactone, the concentrations of 17OHP by both assay methods increased parallel to each other and exceeded the cutoff level, but returned to normal once spironolactone was withdrawn. By contrast, 17OHP concentration, measured by direct assay in kit E before treatment, was noticeably increased, although the extraction assay value was within normal limits. This initial increase in 17OHP by kit E was considered to be due to some hydrophilic steroid secreted in premature infants. Using kits E and C-3 (data not shown) following extraction assay, all 17OHP values remained within the normal range, and their

Figure 2 Clinical course of case 1 showing correlation between spironolactone and 17OHP concentration.

time course changes paralleled those obtained with kit C-7.

Concentrations of spironolactone metabolites in a CAH false positive case (case 1) were measured by HPLC using blood spots on filter paper. Although 17OHP was below the detectable level, spironolactone and its major derivatives, 7 α -thiomethylspironolactone, canrenone, and 6 β -hydroxy-7 α -thiomethylspironolactone, were detected at 32, 575, 153 and 132 ng/ml blood, respectively. This indicates that nearly 900 ng/ml in gross concentration of spironolactone and its metabolites were present in the patient's blood.

From 1 April 1996 to 31 March 1997, the total number of tested cases for CAH was 33938. The number of cases identified as false positive was 235, of which 29 (12.3%) were found to have been treated with spironolactone. Fourteen of the 29 cases had congenital heart disease, and the remaining 15 cases were mostly low birthweight infants.

Figure 3 Clinical course of case 2 showing that 17OHP values were consistently high during treatment with spironolactone and fell once treatment was stopped.

Figure 4 Clinical course of case 3 showing correlation between concentrations of 17OHP and treatment with spironolactone.

Discussion

Determination of 17OHP in dried blood spots by ELISA is used as the initial screening test for CAH. Although the assay is sensitive and reproducible, the specificity is not sufficient to differentiate true positive patients with CAH from false positive cases associated with prematurity^{1-5 11} and/or stress.^{12 14} In general, aside from the above cited causes, relatively high 17OHP values of unknown origin were often observed as most were noted to be transitory. It has been suggested that false positivity under these circumstances could be related to certain drugs, including steroids, which immunologically cross react with 17OHP in the assay system. This report documents that, at least in some of the false positive cases, the cause is due to spironolactone, which cross reacts with 17OHP in the assay. With kit C-7, 17OHP exceeded the cutoff level when the babies were administered therapeutic amounts of spironolactone and returned to normal when the drug dose was reduced or withdrawn. In contrast, assays using kits E or kit C-3, did not detect the false positive cases due to spironolactone, whereas premature babies or babies with severe stress showed false positive test results in direct

assay, possibly due to a cross reaction with hydrophilic steroids.

A gross concentration of spironolactone and its metabolites (900 ng/ml) was detected by HPLC in the blood on filter paper from a patient who had been treated with spironolactone. This observation is compatible with a previous report indicating that the total serum concentration of spironolactone and its metabolites reaches 1000 ng/ml several hours after administration of a single oral dose of 200 mg spironolactone given to men weighing 65–87 kg (mean dose: 2.6 mg/kg weight).¹⁵ Ordinarily, the percentage of cross reactivity less than 0.01% between 17OHP and spironolactone and its metabolites in kit C-7 is negligible. However, in this study an actual additive effect of spironolactone on the value of 17OHP was observed with the drug blood concentration at more than 10 ng/ml (fig 1). This indicates that if the blood concentration of spironolactone exceeds 10 ng/ml, it seems to affect the 17OHP concentration, especially in the extraction assay, where lipophilic compounds such as spironolactone and its metabolites, canrenone and sulphur containing intermediate metabolites,²⁰ are preferentially extracted.

In almost all cases tested with kits E and C-3 using the extraction assay, including the three patients reported above, the time course changes of the apparent 17OHP concentration paralleled those obtained with kit C-7, although the values obtained with the former two kits remained within the normal range. This observation indicates that spironolactone and its metabolites also interfere with 17OHP concentration, to some extent, in kits other than kit C-7. There may be other kits with characteristics similar to those of kit C-7, and this should be made known to laboratories using 17OHP neonatal screening kits.

Spironolactone and its metabolites, canrenone and canrenoate, can inhibit 11 β -hydroxylase and 18-hydroxylase, possibly leading to the increase in blood concentration of 17OHP.^{21–24} However, this hypothesis seems unlikely as assay kits other than kit C-7 did not exhibit high concentrations of 17OHP in cases receiving spironolactone. In our study, about 12% of the false positive cases were identified as being due to the administration of spironolactone.

The above mentioned phenomena had not been identified at the developmental phase of this kit, but were found for the first time after a long time in use. Of course, the ELISA for CAH neonatal screening by kit C-7 is not used worldwide. However, every antibody used in currently available kits for CAH neonatal screening worldwide is not strictly specific for 17OHP. Reduction of the false positive rate definitely depends on antibody specificity but not on the procedures such as EIA (enzyme immunoassay), FIA (fluoroimmunoassay), or RIA (radioimmunoassay), which are used worldwide. In this context, there must be situations where certain drugs or substances present in blood at high concentrations may interfere with the assay and lead to false positive screening results.

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