tained for the other three products between our results and the purported concentrations of II. Product D contains pramoxine hydrochloride, which had a retention time on HPLC that overlapped with II, precluding the determination of II in this product.

Table II presents the percent recovery of I and II from each of the products as determined without the addition of the internal standard, phenyl salicylate. Approximately 58–98% of I was recovered, while 63–91% of II was recovered.

In summary a simple, precise, and accurate HPLC method has been developed that can be used for routine analysis and quality control for iodochlorhydroxyquin and hydrocortisone in creams and ointments.

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Urinary Excretion of Methylparaben and Its Metabolites in Preterm Infants

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Received June 15, 1982, from **Foundation 41, Foetal Pharmacology Laboratory*, and the ‡*Neonatal Unit, The Womens Hospital, Sydney, N.S.W. Australia 2010.* Accepted for publication August 6, 1982.

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Abstract □ A high-performance liquid chromatographic (HPLC) assay to quantitate methylparaben in urine was developed. Standard curves were linear and recovery of the paraben from urine averaged 82.6%. The urinary excretion of methylparaben in six preterm infants (≤31 weeks gestational age), who were receiving intramuscular injections of a paraben-containing gentamicin formulation, ranged from 13.2 to 88.1%. Small quantities of the metabolite, *p*-hydroxybenzoic acid, were detected by GC–MS.

Keyphrases □ Methylparaben—determination in human urine, preterm infants, high-performance liquid chromatography □ High-performance liquid chromatography—methylparaben in human urine, preterm infants, gentamicin formulations □ Preservatives—methylparaben, determination in human urine, preterm infants, high-performance liquid chromatography

Methylparaben (methyl *p*-hydroxybenzoate) and propylparaben (propyl *p*-hydroxybenzoate) are used in combination (in some medications) as preservatives. Reports that methylparaben can displace bilirubin from its binding sites on albumin *in vitro* has produced some concern regarding its use in medicinal formulations administered to the newborn and, in particular, the preterm infant (1–3). Displacement of bilirubin from its binding sites in infants with neonatal jaundice could lead to brain damage (kernicterus).

Gentamicin, an effective antibiotic for treatment of a neonatal Gram-negative sepsis, was also suspected of being a bilirubin displacer (4). Subsequent experiments have proven that gentamicin does not displace bilirubin (2, 5). However, some gentamicin formulations do contain methylparaben as a preservative. In an *in vivo* study a group of infants received a single intramuscular injection of a paraben-containing gentamicin formulation. No reduction in the albumin binding capacity for bilirubin was detected (3). The differences seen between this *in vivo* study and the *in vitro* studies may have been due to *in vivo* preservative catabolism or low paraben serum levels achieved after intramuscular injection. The concentrations of methylparaben used *in vitro* were relatively high, *e.g.*, 167 μg/ml of serum (2).

In this study, the excretion and metabolism of methylparaben was monitored in preterm infants after they had received multiple doses of a gentamicin formulation containing paraben preservatives.

EXPERIMENTAL

Materials—Methyl-, propyl-, and *n*-butylparabens[1], phosphoric acid[2], glacial acetic acid[3], and sodium hydroxide[4] were obtained commercially, and appropriate aqueous or methanolic solutions were prepared as required. Urine samples were extracted with freshly glass-distilled ether[5]. An acetate buffer (100 ml, pH 5.0) was prepared by mixing 0.2 *M* acetic acid (14.8 ml), 0.2 *M* sodium acetate[2] (35.2 ml), and water. A 1 *M* carbonate buffer (pH 9.5) was prepared by adding sodium carbonate[2] (5.3 g) and sodium bicarbonate[2] (4.2 g) to water (100 ml). Gentamicin[6] was obtained commercially; each vial (2 ml) contained gentamicin (80 mg), methylparaben (3.6 mg), and propylparaben (0.4 mg).

Patients—Six preterm babies (4 males, 2 females), with estimated gestatinal ages of 26–31 weeks and birth weights of 0.69–1.50 kg, were studied. Each infant was admitted to the neonatal intensive care unit and

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Ajax Chemicals Ltd., Sydney, Australia.
[3] B.D.H. Chemicals (Aust) Pty. Ltd., Fairy, Vic., Australia.
[4] E. Merck, Darmstadt, West Germany.
[5] Anesthetic ether B.P.; Hoechst Australia Ltd., Melbourne, Vic., Australia.
[6] Gentamicin Injection, B.P.; David Bull Lab. Pty. Ltd., Mulgrave, Vic., Australia.

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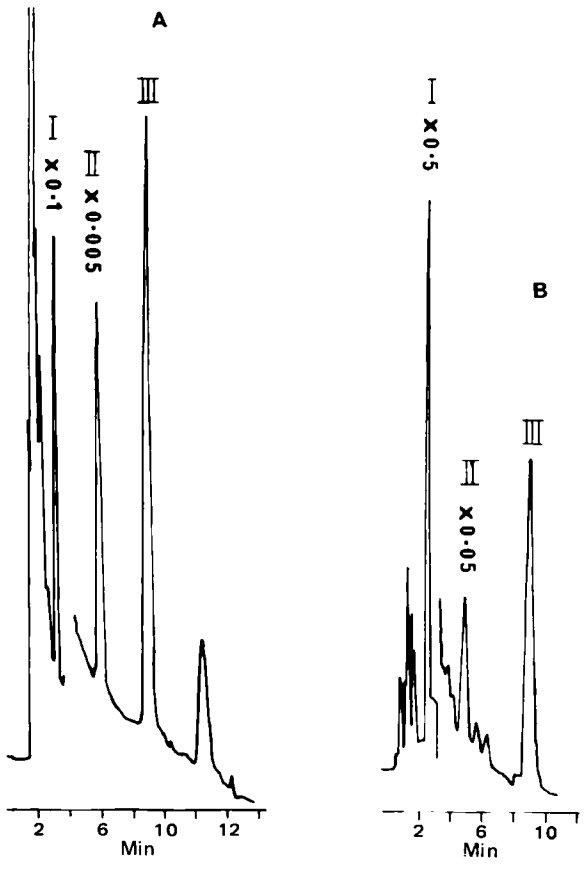

Figure 1—*Chromatograms (HPLC) of urine extracts. Key: (A) urine sample spiked with methylparaben (I), propylparaben (II), and butylparaben (III); (B) urine from a subject receiving a paraben-containing gentamicin formulation. Key: (I) methylparaben, (II) an endogenous substance, (III) butylparaben.*

was being treated for suspected sepsis with intramuscular gentamicin, 2.5 mg/kg every 18 hr or 3.0 mg/kg every 24 hr. Each infant was given gentamicin from the first day of extrauterine life. For infants on the 2.5-mg/kg dosage regimen, urine collection was 18 hr in duration and thus spanned the dosage interval. When the infant voided, time and volume were recorded and the collecting device[7] was replaced. With the two infants on the 3.0-mg/kg dosage regimen, urine was collected only when the collecting device was filled, and the total 24-hr urine was pooled prior to analysis. None of the infants were on any other drugs containing methylparaben as a preservative. Four of the infants were receiving electrolytes, vitamins, and amino acid nutrition (hyperalimentation, no paraben preservatives). The other two infants were on their first day of pooled breast milk.

Extraction of Urine Samples—Aliquots (1–2 ml) of urine from each collection period were diluted with an equal volume of acetate buffer and incubated in a water bath[8] at 37° for 18 hr with β-glucuronidase[9] (5 mg) and sulfatase[10] (5 mg). *n*-Butylparaben (800 mg) was added as the internal standard, and the hydrolyzed urine was adjusted first to pH 9 for extraction of methylparaben using carbonate buffer (400–600 μl) and then to pH 2 for extraction of metabolites with phosphoric acid. The urine samples (pH 9) were extracted[11] twice with ether (5 ml) for 1 min, and then centrifuged[12] at 1000×*g* for 5 min. The organic layers were back-extracted into a 0.1 *M* NaOH solution (300 μl) and the ethereal layer was discarded. The basic solution was acidified (pH 2) with phosphoric acid (1 drop) and subsequently extracted with ether (5 ml). After centrifugation (1000×*g*) the ether was transferred to a glass evaporation tube. A tapered pipet[13] with a sealed end was inserted and the tube placed in a 40° water bath[14]. Immediately after evaporation the tubes were stoppered and placed in an ice bath to allow the ether to condense and wash down the inner walls of the evaporation tube. The condensed ether was evaporated under a nitrogen stream. Before injection into the liquid chromatograph, the residue was reconstituted in 200 μl of mobile phase by mixing[11] (30 sec).

After extracting at pH 9, urine samples were adjusted to pH 2 and extracted twice with ether (5 ml) in a similar fashion to that described above. The ether was evaporated and the residues derivatized utilizing *N,N*-dimethylformamide di-*n*-butyl acetal[15] (20 μl) and acetonitrile[1] (10 μl; silylation grade). The stoppered tubes were heated (water bath, 48°) for 15 min before injection into the gas chromatograph–mass spectrometer.

High-Performance Liquid Chromatography (HPLC)—Methylparaben concentrations were determined utilizing a liquid chromatograph[16] equipped with a loop injection system[17] and a 30 cm × 3.9-mm (i.d.) octadecylsilane column[18]. A UV detector[19] equipped with a 254-nm filter and a dual-pen recorder[20] (with inputs set at 10 mV and chart speed of 20 cm/hr) was used. The mobile phase, HPLC-grade acetonitrile[21] and 0.05% phosphoric acid (40:60) was pumped through the column at 90 ml/hr, resulting in an inlet pressure of 900 psig.

Gas Chromatography–Mass Spectrometry (GC–MS)—GC–MS was performed on a GC–mass spectrometer equipped with a data system[22]. The chromatographic column was a 0.9 m × 2-mm (i.d.) coiled, glass column packed with 3% OV-17 on 100–120 mesh Gas Chromosorb Q[23]. The injection port temperature was 240°, and the helium flow rate 30 ml/min. The column was heated at 160° (1 min) and then increased to 220° (2 min) at a rate of 10°/min. A solvent wait period of 0.5 min was employed.

Methylparaben concentrations were calculated from standard curves made after extracting known quantities of methylparaben which had been added to urine. A standard curve was plotted as the peak height ratio of methylparaben to internal standard (butylparaben) against known methylparaben concentrations. Creatinine levels were determined using the Jaffe kinetic reaction[24].

Table I—Urinary Excretion of Methylparaben

Patient	Amount Administered, μg	Total Amount Detected, μg	Percent of Dose Excreted
A	70.0	9.24	13.2
B[a]	170.0	27.7	16.3
C	85.0	74.9	88.1
D	135.0	21.7	16.1
E[a]	190.0	67.9	35.7
F	160.0	54.6	34.1

[a] Urine collected over 24 hr.

RESULTS AND DISCUSSION

The gentamicin administered intramuscularly to the infants in this study contained both methyl- and propylparaben. HPLC analysis of spiked urine extracts gave two well-defined peaks adequately separated from the internal standard, butylparaben (peak III, Fig. 1A). Peaks I and II (Fig. 1A) are methyl- and propylparaben, respectively. The urine extracts from the infants in this study showed, on analysis, one major peak (peak I, Fig. 1B) other than the internal standard (peak III) which was not present on analysis of a control urine extract. The solvent eluant corresponding to the retention time of peak I (Fig. 1B) was collected, extracted, and identified by GC–MS as methylparaben (molecular ion, *m/z* 152). Peak II (Fig. 1B) was due to an endogenous substance. Although it was not the intention of this study to measure propylparaben levels,

[7] U-Bag, newborn size; Hollister Inc., Chicago, Ill.
[8] Gared Thermoline Pty. Ltd., Smithfield, N.S.W. Australia.
[9] No. G-0251, Type B-1; Sigma Chemical Co., St. Louis, Mo.
[10] No. S-8504, from limpets *Patella vulgata*, Type IV; Sigma Chemical Co., St. Louis, Mo.
[11] Vortex-Genie; Scientific Industries Inc., Springfield, Mass.
[12] *GS-200 centrifuge*; H. J. Clements Pty. Ltd., Sydney, Australia.
[13] Disposable Pasteur pipets (9″); Lab. Supply Pty. Ltd., Sydney, Australia.
[14] Ken Lab WBC_6; Townson & Mercer Distributors Pty. Ltd., Sydney, Australia.
[15] *n*-Butyl 8; Pierce Chemical Co., Rockford, Ill.
[16] Varian Aerograph 8500 Liquid Chromatograph; Varian Instruments, Palo Alto, Calif.
[17] Valco, Houston, Tex.
[18] C_{18} μ-Bondapak column; Waters Associates Inc., Milford, Mass.
[19] Model 440 ultraviolet detector; Waters Associates Inc., Milford, Mass.
[20] Model 585; Cole Parmer, Chicago, Ill.
[21] Waters Associates Inc., Milford, Mass.
[22] Model 5992 GC/MS system controlled by a Flexible Disc Software using the HP Model 9825 Desk Computer and HP 9885 Flexible Disc Drives; Hewlett-Packard, Avondale, Pa.
[23] Applied Science, Milton Roy Co., State College, Pa.
[24] Smith Kline Co., Sydney, Australia.

this paraben was detectable, and verified by GC–MS (molecular ion, m/z 166) in the pooled 24-hr urine extracts. The samples obtained at the time of urine void during the 18-hr collection period were not of sufficient volume for propylparaben analysis. The standard curve for the HPLC analysis of methylparaben was linear (r^2, coefficient of determination, 0.996) over a concentration range of 100–2000 ng/ml. The mean recovery of methylparaben from urine was 82.6 ± 4.9% (SD).

The percentage of the methylparaben dose accounted for in hydrolyzed urine samples (collected over 18 or 24 hr) after gentamicin administration varied from 13.2 to 88.1% (Table I). Of the methylparaben excreted in the urine the majority was in conjugated form. Extraction of nonhydrolyzed urine samples yielded <1% methylparaben. Analysis of a single urine sample divided into three aliquots and hydrolyzed with sulfatase, β-glucuronidase, or β-glucuronidase in combination with sulfatase indicated sulfate conjugates were more abundant (19% greater) than glucuronides. Levy *et al.* (6) noted the decreased phenolic glucuronidation of acetaminophen was partially compensated, in the full-term neonate, by a well-developed capacity for sulfate conjugation.

A number of factors may be responsible for the poor recovery of methylparaben in most of these infants. The patients may not have achieved a steady state with respect to parabens. However, each infant had been receiving gentamicin from the first day postpartum. The volume of distribution is increased due to a greater total water content in the newborn and a higher ratio of extracellular to intracellular water (7). Extracellular fluid in the newborn may be as high as 45% (8). The maximum urinary excretion rate of methylparaben in the infants varied, ranging from 1.5 to 17 hr (A, 4.5 hr; C, 6.25 hr; D, 1.5 hr; F, 17 hr). This variability may, in part, be due to differences in intramuscular absorption. Peripheral vasomotor instability, changes in relative blood flow of muscles (due to maturational adaptation), and relative insufficiency of muscular contractions account for variation in intramuscular absorption in the newborn (7).

One patient (C) who was 11 days of age excreted 88.1% of the administered methylparaben (Table I). Serum creatinine determinations (Table II) indicated renal function was adequate. Only two infants had creatinine levels within the normal range (<0.10 mmoles/liter). An improvement in glomerular filtration is usually seen within the first few days of the preterm infants' extrauterine life (7).

Methylparaben may be eliminated by routes other than renal excretion and/or metabolized by means other than conjugation. The only metabolite detected was p-hydroxybenzoic acid. GC–MS analysis of a pH 2, butylated, hydrolyzed urine extract revealed a small peak (R_T, 3.1 min) which gave prominent ions at m/z 194, 138, and 121. These latter ions were due to the loss of a C_4H_8 molecule and a C_4H_9O radical, respectively, from the molecular ion of n-butylparaben, m/z 194. The retention time and mass spectral data were identical to those obtained on GC–MS analysis of an authentic sample of p-hydroxybenzoic acid which had been treated with N,N-dimethylformamide di-n-butyl acetal. The recovery of this metabolite was not attempted because the peak was small and in some cases appeared only as a shoulder on an endogenous urine peak. Perhaps the small recovery of this metabolite is not surprising since esterase activity in preterm infants is low (8).

Glycine conjugation is evident in hepatic, renal, and intestinal tissue at relatively early stages of gestation (9). The glycine conjugate of p-hydroxybenzoic acid was not detected, but hippuric acid (glycine conjugate of benzoic acid) was seen in the butylated pH 2 urine extracts. GC–MS analysis of these extracts revealed a peak (R_T, 4.1 min) with a molecular ion at m/z 235 and prominent ions at m/z 134 and 105 due to the loss of $C_5H_9O_2$ and $C_6H_{12}NO_2$ fragments, respectively, from the molecular ion. p-Hydroxyhippuric acid, the glucuronide of p-hydroxybenzoic acid, and the ether sulfate of p-hydroxybenzoic acid were the main metabolites detected in the urine and bile of the rat after the administration of ethylparaben (10).

Table II—Patient Data

Patient	Birth Wt., kg	Gestational Age, weeks	Postpartum Age, days	Serum Creatinine, mmoles/liter
A	0.69	26	3	0.16
B	1.20	27	3	0.05
C	0.83	27	11	0.03
D	1.19	30	3	0.13
E	1.45	30	7	0.13
F	1.50	31	3	0.13

Although the number of infants studied is small, recovery of methylparaben appears to improve as gestational and postpartum age increase. Excluding patient C (discussed earlier), the recoveries were greatest in patients E and F (Table I). Both of these infants were 31 weeks conceptional (gestational plus postnatal) age.

In conclusion, the excretion of methylparaben by the urinary route, in preterm infants is variable during the first few days of extrauterine life. These infants are often on medications, such as gentamicin, that contain paraben preservatives for extended periods of time. Whether there is an accumulation of the preservatives in the body and whether after repeated injections the albumin binding capacity for bilirubin is affected remains to be determined. It is not uncommon for preterm infants to have neonatal jaundice. In fact, except for patient F the preterm infants in this study had jaundice which was treated with phototherapy. The differences seen between the *in vitro* and *in vivo* studies (1–3) cannot be explained by preservative metabolism or urinary excretion in the preterm infant as postulated by Woods *et al.* (3).

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ACKNOWLEDGMENTS

The assistance of the Medical Research Council in the form of travel assistance (for K.W.H.) is appreciated.

Dr. Roger Nation is gratefully acknowledged for helpful discussions.