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6-Sulfatoxymelatonin Collected From Infant Diapers: Feasibility and Implications for Urinary Biochemical Markers

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Abstract

The purpose of this study was to assess feasibility and acceptability of using a diaper pad for collection of in-home infant urinary samples and to test the accuracy of diaper pad extraction for 6-sulfatoxymelatonin and creatinine, which was used to correct assay results for urinary volume. To assess feasibility and acceptability, urine samples from 20 infants were collected over a 24-hour day using a cotton pad inserted in the diaper. The accuracy of diaper pad extraction was evaluated in the laboratory setting using urine samples collected from 11 adult volunteers and assayed using enzyme immunosorbent assay (EIA). Urine samples were divided, one aliquot was assayed without extraction and one aliquot was instilled into a diaper pad, extracted, and assayed. Mothers found diaper pad collection acceptable and easy to perform. Of 144 infant urinary samples obtained in the home environment, 59% were usable for assay purposes, the remaining either were contaminated with stool or were of insufficient volume. While creatinine values from diaper pad extracted and non-extracted samples were highly correlated ($R^2 = 0.947$) those of creatinine corrected 6-sulfatoxymelatonin were not ($R^2 = 0.216$). Diaper pad collection procedures altered 6-sulfatoxymelatonin values. Implications for measurement of urinary biochemical substances and statistical analysis are discussed.

Keywords

infant; urine; methods; biological assay

Introduction

The purpose of this research was to test the feasibility of obtaining serial urinary samples from infant diapers to measure 6-sulfatoxymelatonin throughout a 24-hour day in the home environment and to test the accuracy of a procedure for extracting urine samples from diapers. Findings illustrate research issues in collecting urinary specimens from infants, the need for careful evaluation of data prior to statistical comparison, and alteration of assay results by collection procedure.

Neonatal and infant nurse researchers are challenged by the need for non-invasive measures of endocrine and biochemical variables. Although salivary measures are well suited as non-invasive measures, the repeated sampling required for variables demonstrating biological rhythm is difficult to implement. Further, serial salivary sampling is demanding when the study protocol involves data collection performed by parents in the home environment.

Endocrine and biochemical variables obtained from urine may be a reasonable alternative. Episodic samples of urine, following the typical voiding pattern, reflect the time period from last voiding, and thus are not well suited for singular measurement at a precise time point. For infants who cannot void on demand, urine present in the diaper reflects urine produced since the last diaper change. Repeated sampling of urine across time, however, can be used to determine biological rhythm including cycle mesor (mean), amplitude, and acrophase as well as area under the curve (AUC) representing total value across a time period (Thomas & Burr, 2006).

Collection of infant urine samples for research as well as for practice can be achieved in several ways. Choice of approach is also guided by the type of sample desired, such as complete collection of all urine over a 24-hour period, single isolated urine sample, or serial episodic samples collected over a specified time period. Sampling approaches that preserve volume of urine (e.g., a 24-hour sample or single voiding in which volume is recorded) allow the measured substance to be expressed as amount per ml of urine. Testing an aliquot of urine from a voiding requires correction when volume is unknown because glomerular filtration rate, and therefore urinary volume, differs over time. Substances measured from aliquots of urine where the total volume of the voiding is unknown are typically corrected using creatinine (amount of substance/ml divided by mg creatinine/ml) and expressed as amount/mg of creatinine (Klante, Brinschwitz, Secci, Wollnik, & Steinlechner, 1997).

Procedures for infant urinary collection present advantages and disadvantages. Urine samples may be obtained using urinary collection bags adhered to the infant's perineum. Isolated samples may be collected using one-time use collection bags, or collection bags with drainage tubes allowing multiple samples over a 24-hour sample may be employed (Commentz, Uhlig, Henke, Hellwege, & Willig, 1997). Limitations of collection bags include adequate adherence to the skin and potential stripping of epithelium and discomfort with bag removal. Alternatively, diapers can be collected and urine samples extracted from the diaper (Burke, 1995; Heckmann et al., 2005). If the diapers are weighed before and after placement on the infant, urine volume can be determined. This approach requires disposable diapers and may involve either emulsifying the diaper as a whole (Kennaway, Stamp, & Goble, 1992), expressing urine from a saturated area of the diaper material, or chemically extracting urine (Hu, Beach, Raymer, & Gardner, 2004). Emulsifying whole diapers is time consuming, resource intense, and raises biohazard concerns. Expression of urine from a segment of the diaper (typically by placing a piece of the saturated diaper material in a syringe barrel then inserting the plunger and expelling the sample or other source of pressure) is problematic with gel-based absorbent diaper material which are an industry standard. Chemical extraction adds expense and the possibility of altering the substance of interest present in the urine.

As an alternative to collecting whole diapers or expressing urine from a portion of the diaper, an absorbent pad (cotton, gauze, etc.) can be placed in the diaper over the infant's urinary meatus (Burke, 1995; Dorey & Zimmermann, 2008; Macfarlane, Ellis, Hughes, Houghton, & Lord, 2005; Rao, Bhatt, Houghton, & Macfarlane, 2004; Sadeh, 1997). The pad is collected at time of diaper change and stored in a urine specimen container. Only the pad is retained and urine is then expressed from the pad using a pressure source as described above, tested, and values corrected for urinary volume using creatinine (Klante et al., 1997). This collection strategy is easily employed, requires modest effort and limited instruction and is frequently used in clinical practice. The diaper pad collection system is straight forward, but as with all diaper urinary extraction approaches, it is essential that the specimen not be altered by the diaper or pad materials or the extraction process.

As with all urinary collection approaches in diapered infants, contamination by stool is also a consideration. The biochemical substance of interest may be present in stool, as well as in

urine, altering assay results. Additionally stool contents may interact with the substance of interest invalidating assay findings. Finally, in designing studies involving collection of infant urine it is important to anticipate the proportion of infant diapers that contain stool.

While findings from the current study inform collection of urine for biochemical measurements in general, the specific study variables were 6-sulfatoxymelatonin and creatinine. Melatonin is a marker of photoentrainment and circadian rhythm (Kennaway & Wright, 2002). Urinary 6-sulfatoxymelatonin, the primary metabolite of melatonin, closely parallels serum values and directly reflects pineal production (Matthews, Guerin, & Wang, 1991). Creatinine, which is freely diffusible in the renal glomerulus, was used to correct diaper pad samples for urinary volume (Klante et al., 1997).

The study reported here investigated the feasibility of collecting serial urinary samples using the diaper pad approach and the accuracy of urine extraction from diaper pads. Specific aims included: 1) Determine total number of diapers collected per day, proportion of urine samples contaminated with stool, and proportion of retrievable urine samples, and 2) Compare 6-sulfatoxymelatonin and creatinine assay results from urine collected with and without diaper pad extraction. Secondly, the project tested in-home procedures for urine collection and maternal acceptability was evaluated.

Materials and Methods

Design and Sample

Two groups of subjects were studied. For in-home testing of collection procedures, twenty healthy infants (age M 58.7 (SD 10.6) days) and their mothers were studied using a descriptive and exploratory design. Subjects were recruited through parenting classes and advertisements, and infants met the following inclusion criteria: uncomplicated antenatal and postnatal history, term gestation, vaginal birth, and four to ten weeks postnatal age. The accuracy of diaper pad extraction of urine was tested using urine samples collected from 11 anonymous adult volunteers who provided a one-time urine sample. Sample size for the comparison of assay accuracy was determined by power analysis with moderate effect size, $\alpha \leq 0.05$, and $\beta = 0.85$ using estimates of infant minimum and mean 6-sulfatoxymelatonin (Ardura, Gutierrez, Andrés, & Agapito, 2003).

Instruments

Diary—Mothers participating in the in-home portion of the study completed a 24-hour diary, divided into 15 minute epochs, and indicated infant diaper changes and collection of urine samples. Urine pads collected were checked against the maternal diary to assure completeness of sample collection.

Acceptability—At the end of data collection mothers rated ease of collecting infant urine samples on a 5-point Likert scale (very easy to very hard) survey item.

6-sulfatoxymelatonin—Urine samples were batch assayed using EIA (ALPCO Diagnostics, Salem, NH), range 0.8–40 ng/ml, sensitivity 0.35 ng/ml, inter-assay variability 10.7%, intra-assay variability 8.2%.

Creatinine—Urine samples were also batch assayed for creatinine using high performance liquid chromatography (HPLC), intra-assay variation 5.4%, inter-assay variation 8.4%, standard curve 5 – 200 mg/ml.

Urine assays—For the assays, 5 µl of urine were required for each sulphatoxymelatonin and creatinine. A minimum of 3 ml of unprocessed urine was established as minimal sample size for laboratory procedures. Samples were centrifuged prior to pipetting into assay plate wells and samples were run in duplicate. Remaining sample volume was retained for possible repeat assay.

Procedure

The project was approved by the institutional review board. For the infant sample, a home visit was conducted and informed consent was obtained. The mother was instructed in use of the diaper pad, collection of specimens, and storage of specimens. With each diaper change over a 24-hour period, the diaper pad was removed and placed in a separate sealed plastic bag. Mother's labeled the individual bags containing the pad with date and time of collection and then placed samples in a collection bag which was stored in the freezer compartment of the home refrigerator. The diaper change and specimen collection were recorded in the diary. Samples were collected on a return home visit and transported on ice in a cooler to the laboratory setting. In preparation for assay, the urine pads were placed individually in a 5cc syringe, urine was expressed into a specimen vial containing disodium EDTA/sodium metabisulfite preservative and stored at -70°C .

Procedures for determination of the accuracy of diaper pad extraction included anonymous adult volunteer donations of urine which were collected on one day and stored on ice for up to four hours. Each urine sample was divided, an aliquot was placed directly into a specimen vial, and a second aliquot was used to saturate a diaper pad which was then prepared for assay and stored as above. Saturated diaper pads contained 15 ml of urine. The same 100% cotton pad (Iris, Montreal, CN) was used throughout the project.

Analysis

Descriptive statistics were used to summarize results from the collection of infant specimens. Analysis plans assessing accuracy of diaper pad extraction entailed plotting the data, comparison and correlation of extracted and non-extracted assay values for 6-sMT and creatinine using paired t-test and Pearson correlation, respectively.

Results

There were a total of 144 diaper pad samples collected from the 20 infant subjects. A description of sample usability is provided in Table 1. Fifty-nine percent of the infant diaper pad samples were ultimately assayable. Stool contaminated 23% of samples and in 18% there was insufficient volume for assay (i.e., < 3 ml of urine were obtainable from the diaper pad).. Approximately 4–5 usable samples were obtained per infant. Mean maternal acceptability rating was 4.33 ($SD = 0.73$; 5 = Very Easy).

The accuracy of assays results obtained with and without diaper pad extraction raised concerns. Initial scatter plots (Figure 1) showed that while the correlation between the diaper pad extracted and non-extracted creatinine was very high ($r = 0.973$, $r^2 = 0.947$), the relation for uncorrected 6-sulfatoxymelatonin was remarkably low ($r = 0.465$, $r^2 = 0.216$). A relation between urinary creatinine and uncorrected 6-sulfatoxymelatonin values would be expected because both values are related to glomerular filtration rate and urine volume. However further analysis revealed that the diaper pad extraction altered this relation (Figure 2). Correlation between creatinine and uncorrected melatonin was $r = 0.836$ ($r^2 = 0.699$) without extraction and $r = 0.431$ ($r^2 = 0.186$) with extraction. Figure 2 further illustrates an interaction effect in that the relation between uncorrected 6-sulfatoxymelatonin and creatinine is not the same in diaper pad extracted vs. non-extracted samples. Although the interaction observed in the scatter

plots does not support application of paired t-test, this analysis was performed for illustration purposes. Paired t-test results showed no statistically significant differences in creatinine, corrected and uncorrected 6-sulfatoxymelatonin, however this finding must be viewed in light of the observed interaction. Sample size did not allow statistical evaluation of this interaction using a multivariate approach.

Discussion

Mothers reported high acceptability for diaper pad urinary collection system used in the home environment similar to findings in the literature (Hu et al., 2004; Liaw, Nayar, Pedler, & Coulthard, 2000). Accounting for stool contamination and limited saturation of the pad, fifty-nine percent of collected diaper pad samples ultimately were assayable. For each infant, on average roughly 4–5 diapers per day provided assayable samples and 6 of 20 infants (30%) had fewer than 4 assayable samples per day. Urinary biological markers have a significant time-of-day effect and missing samples limit the ability to portray a 24-hour period. Therefore, while acceptable, the diaper pad infant urine collection system did not reliably yield a sufficient number of samples.

Testing of diaper pad extraction accuracy showed that creatinine values were not altered by the procedure unlike 6-sulfatoxymelatonin values which were. Explanations for the effect of diaper pad extraction on 6-sulfatoxymelatonin include chemical reaction of 6-sulfatoxymelatonin with pad material or specificity of the assay (e.g., influence of other substances in the urine).

This project provides several valuable lessons, for development of methods for collection and assay of biochemical markers, as well as for analysis of such samples. First, the effect of data collection procedures and sample handling on assay results should be evaluated systematically. Sources of assay measurement error include collection container material, time from collection to processing and storage, temperature during transit and storage. Each procedural step and possible effect on the biochemical substance of interest should be considered carefully. In this particular study the urine extraction procedure was tested using controlled procedures. The difference between extracted and non-extracted urine samples may reflect a chemical interaction between 6-sulfatoxymelatonin and an unknown component of the cotton diaper pad. Other absorbent products could be tested. Second, in comparing procedural effects on assay results, careful consideration of both difference and relation is needed. Ideally the assays of the two groups of samples should demonstrate a small magnitude difference and a very high correlation. In the study reported here, a “quick and dirty” paired t-test of difference between extracted and non-extracted diaper pad samples would have led to the conclusion that the extraction process produced accurate results, yet the moderate correlation between extracted and non-extracted 6-sulfatoxymelatonin was highly suspicious. Third, results that do not follow expectations should be pursued. In this case, the anticipated relation between creatinine and uncorrected 6-sulfatoxymelatonin was not observed and plotting revealed an interaction effect. Fourth, results illustrate the effect of statistical interaction. Although predicted power was adequate for a simple comparison, the power was not based on possible interaction.

In summary, mothers found home collection of infant urinary samples using an absorbent pad placed in the diaper acceptable. Stooling and low urine volume limit the number of infant urine samples that can successfully be collected over a 24-hour period. In the home environment, separation of stool and urine in a diaper is not feasible, particularly given the consistency of infant stools. The accuracy of 6-sulfatoxymelatonin extracted from diaper pads was unacceptable while that for creatinine, used to correct for urine volume, was acceptable.

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Figure 1. Scatter plots of urinary creatinine and uncorrected 6-sulfatoxymelatonin (6-sMT) showing the relation between samples extracted from diaper pads and non-extracted samples ($n = 11$).

Figure 2. Scatterplot of urinary uncorrected 6-sulfatoxymelatonin (6-MT) and creatinine for extracted and nonextracted samples ($n = 11$).

Table 1

Urine sample recovery from 144 diaper pad samples collected from 20 infants over 24-hours.

Variable	<i>M (SD)</i>	Min/max	fr (%) ^a
Diapers collected per infant	7.2 (1.8)	4/10	-
Stool present per infant	2.5 (1.7)	1/7	33 (23%)
Adequate sample for assay per infant	4.5 (2.1)	1/10	85 (59%)

^aFrequency and percent of 144 diapers