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Note

Quantitation of iothalamate in serum and urine by high-performance liquid chromatography

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Assessment of the glomerular filtration rate (GFR) is useful in managing certain critically ill patients, in evaluation of renal function in patients with suspected kidney disease, and to define the role of renal function on drug disposition. The standard method of evaluating GFR is by measuring the renal clearance of endogenous creatinine [1—3]. This process is subject of permutation by ingested creatinine, has a diurnal variation, and varies with the degree of muscular exertion. Renal clearance of creatinine, usually in 24 h, depends on the quantitation of the metabolite by a relatively specific, colorimetric assay [4].

Certain xenobiotics are not metabolized and are cleared exclusively by renal excretion. Iodinated aromatic compounds used as contrast reagents for intravenous angiography and pyelography are in this class. Clearance of these compounds has been used to assess GFR, and the estimate of this function correlates well with the standard creatinine clearance. Radionuclide-labelled compounds ([^{125}I]iothalamate, for example) were used [5, 6] with the concentration of the drug in serum determined from the contained radioactivity. This method has several disadvantages: repeated administration exposes the patient to radiation, the specific activity changes over short intervals, and special precautions are required to dispose of serum, urine, and equipment containing the radionuclide.

Two high-performance liquid chromatographic (HPLC) assays for

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iothalamate have been developed [7, 8]. One requires large sample volume ($\geqslant$ 0.5 ml) and involves a tedious extraction procedure. The other, recently published, is similar to this method but does not utilize dual-wavelength monitoring.

We developed a rapid and sensitive HPLC assay for iothalamate. This procedure utilizes dual-wavelength monitoring to ensure specificity which uses the common 254 nm detector and more sensitive 229 nm detector. This method also uses Hypaque (iodinated aromatic contrast reagent) as the internal standard.

MATERIALS AND METHODS

Separation was achieved by reversed-phase HPLC. A dual-piston Constametric pump (Laboratory Data Control/Milton Roy, Riviera Beach, FL, U.S.A.) delivered the mobile phase to a 5-μm Radial-Pak C_{18} cartridge (10 cm $\times$ 8 mm I.D.) held under radial compression by an RCM-100 radial compression module (both from Waters Assoc., Milford, MA, U.S.A.). Two fixed-wavelength UV III monitors connected in series measured absorbance of the eluate at 229 nm and 254 nm. A separate Spectral Supply high-voltage power source is required for the 229-nm Cd lamp (all Laboratory Data Control/Milton Roy). The 10-mV signals generated by the detectors were delivered to an Omniscribe B-5217-5 dual-pen strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). Samples were either injected with a Model 7010 sample injection valve fitted with a 20-μl sample loop and Model 7011 loop filler port (Rheodyne, Berkeley, CA, U.S.A.) or 20 μl were injected with a WISP (Waters intelligent sample processor, Waters Assoc.).

Sodium iothalamate comes in the form of a U.S.P. 66.8% (w/v) solution for injection labelled as Conray-400 (Mallinckrodt, St. Louis, MO, U.S.A.). The concentration in each lot of the formulation was determined from its molar extinction coefficient ($E = 3.3 \cdot 10^4$ l/mol/cm at pH 6 at 240 nm.).

Analytical reagent grade Type I water produced by the Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used to prepare all aqueous solutions unless otherwise indicated in the text.

Ultrapure 70% perchloric acid (Alpha Products, Danvers, MA, U.S.A.) was diluted with Type I water to give a 6% (w/v) solution and was used as a serum protein precipitant. The internal standard solution consisted of sodium diatrizoate in a U.S.P. 50% injection (Hypaque from Winthop Labs., New York, NY, U.S.A.) which was diluted with 6% perchloric acid to yield a final concentration of 12.5 μg/ml and was stored at 4°C.

A 4% (w/v) solution of detergent to decrease protein binding of the compound was made by dissolving electrophoresis-pure sodium dodecyl sulfate (BioRad Labs., Richmond, CA, U.S.A.) in Type I water. This was stored in an amber bottle at room temperature.

A 40 mmol/l potassium phosphate buffer, pH 6.0, was prepared from analytical reagent grade monobasic and dibasic potassium phosphate salts (Mallinckrodt) and Type I water. Phosphoric acid and/or aqueous potassium hydroxide were added, if necessary, to achieve pH 6.0. It was filtered using a

0.22-μm MF-Millipore Type GS filter membrane (Millipore) immediately prior to its use in the assay. The buffer was stored in a tightly capped amber bottle at room temperature. This buffer was used within one week.

Iothalamate standards

Fresh standards were prepared daily or aliquots were stored at −70°C. An iothalamate solution of 113.6 μg/ml was prepared in distilled water. A 1:1 dilution of this working standard and distilled water yielded three standards used for this assay with the following concentrations: 113.6, 56.8 and 0 μg/ml. The absorbance of 1:50 dilutions of each standard was measured at 240 nm with an Acta III spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.). This step was used to verify the accuracy of the dilutions.

Sample preparation

Serum (25 μl) was added to 25 μl of 4% SDS solution (25 μl of distilled water were added to aqueous samples). This was mixed and diluted with 50 μl of internal standard solution. This mixture was mixed vigorously for 10 sec and then centrifuged (10,000 *g*) with an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY, U.S.A.) for 3 min. If the supernatant was cloudy, the centrifugation was repeated.

Assay

A 25-μl aliquot of the supernatant was injected into the chromatograph and eluted with the mobile phase at a flow-rate of 3 ml/min at room temperature. Approximate column back-pressure was 124—138 bars. Detector sensitivity was set at 0.016 absorbance units full scale (a.u.f.s.) with a chart speed of 0.5 cm/min. We found that recycling the mobile phase during the assay did not affect resolution or retention times but considerably saved buffer usage. Fresh buffer was used daily. Precolumns with 30—50 μm C_{18} Corasil packing material (Waters Assoc.) protected the analytical column. These precolumns were routinely changed after approx. 100 injections. Columns were washed with filtered Type I water and a mixture of glass-distilled acetonitrile—Type I water (50:50, v/v). When the WISP was utilized, 200 μl of dimethyl sulfoxide were injected when the water wash was employed. This reduced back-pressure and maintained column's resolution. The duration of reliable use of the analytical column varied from 300 to 500 samples.

Calculations

Retention times of iothalamates and internal standard were 10 and 8.25 min, respectively. Chromatograms of an aqueous and a serum sample are depicted in Fig. 1. A standard curve from aqueous standards was determined daily from the ratio of iothalamate:internal standard peak heights and known concentrations. Iothalamate concentrations of unknown specimens were determined from the above standard curve. Specificity was insured by dual-wavelength monitoring. Interferences by other compounds with iothalamate can be detected by a deviation that is twice the standard deviation of the 229/254 nm peak height ratio. The 229/254 nm peak height ratio of iothalamate is 1.11 ± 0.03.

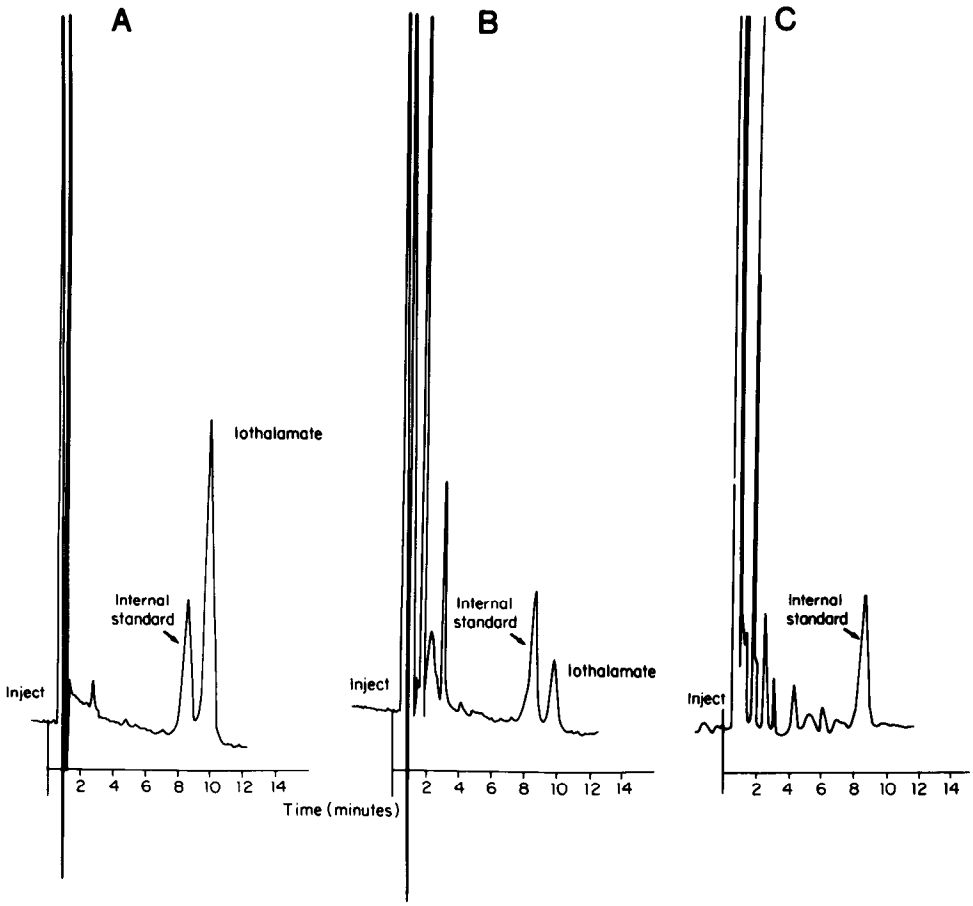

Fig. 1. Chromatogram of (A) an aqueous iothalamate standard, 113.6 μg/ml; (B) a serum sample with calculated iothalamate concentration of 12.5 μg/ml; (C) a serum sample without iothalamate. In all chromatograms, absorbance is at 229 nm with 0.02 a.u.f.s. All samples were prepared as described in Materials and methods.

Linearity

Serial dilutions of iothalamate working standards were made in distilled water to concentrations of 113.6 (no dilution), 56.8, 28.4, 7.1, 0.89, and 0.44 μg/ml. Iothalamate:internal standard peak height ratios were calculated and linear regression was done to calculate the slope, y-intercept and correlation coefficient (r^2).

Precision

Iothalamate was diluted in pooled human serum to three concentrations: 27.5, 50, and 15 μg/ml. Two concentrations, 15 μg/ml (low) and 50 μg/ml (high) were aliquoted and frozen at −70°C. An aliquot for both concentrations was assayed daily which determined run-to-run precision. With statistical analysis, a coefficient of variation was calculated. The 27.5 μg/ml serum solution was assayed ten times on the same day which determined within-run precision. Again, with statistical analysis, a coefficient of variation was calculated.

Accuracy

Recoveries from serum were determined by comparing the peak height ratios of aqueous solutions and serum solutions at the same concentration. Recovery from urine was also determined.

Sensitivity

An aqueous standard was serially diluted with water and assayed until a signal was produced that was twice the background noise. This concentration was defined as lowest sensitivity level of the assay.

Stability

Serum aliquots that were used for precision analysis were stored under four temperature conditions: room temperature (approx. 21°C), 4°C, −20°C, and −70°C. One aliquot was removed from the storage conditions and assayed for iothalamate at zero time, 2, 4, 8, 24, 48, and 72 h, and 1, 2, 4, and 8 weeks.

RESULTS AND DISCUSSION

Linearity

For the concentrations given in Materials and methods (n = 6), a linear regression analysis described the line $y = 0.0319x + 0.0255$ with a correlation coefficient (r^2) of 0.9996. For samples with concentrations greater than 125 μg/ml, dilution to a concentration less than 125 μg/ml was performed.

Precision

Mean values of aliquoted serum samples described in Materials and methods with standard deviations (S.D.) are shown in Table I. Within-run coefficient of variation (C.V.) was 1.58%. The run-to-run coefficients of variation for the high and low concentrations were 3.6% and 5.3%, respectively (n = 25).

TABLE I

PRECISION

Concentrations are given in μg/ml.

	Within-run	Run-to-run	
		High	Low
n	10	25	25
Mean	27.75	50.54	16.19
S.D.	0.43	1.86	0.86
C.V.* (%)	1.58	3.60	5.31

*Coefficient of variation expressed as a percentage of the mean.

Accuracy

Initial mean recoveries of serum were 92% or less. We enhanced recovery by adding 4% (w/v) solution of sodium dodecyl sulfate. The mean recovery improved to 99.8% for serum, while the mean recovery for urine was 99.5%. Measured versus assayed concentrations and recoveries are depicted in Table II.

TABLE II

ACCURACY

Concentrations are given in μg/ml.

Serum			Urine		
Measured concentration	Assayed concentration	Percentage recovered	Measured concentration	Assayed concentration	Percentage recovered
113.6	111.2	97.9	56.8	60.6	106.7
56.8	57.1	100.5	45.4	46.3	102.0
28.4	29.4	103.5	34.1	34.4	100.9
7.1	6.9	97.2	22.7	22.3	98.2
			11.4	12.0	105.3
	Mean =	99.8	56.8	58.4	102.8
			45.4	45.9	100.9
			34.1	32.5	95.3
			22.7	21.3	93.8
			11.4	10.2	89.5
				Mean =	99.5

Sensitivity

The assay was sensitive to 0.3 μg/ml as defined in Materials and methods.

Stability

The stability study at the four different temperatures was conducted for two months. We found no significant difference in the peak height ratios or calculated concentration at any of the temperature conditions after two months of storage as noted. In addition, iothalamate was stable in serum at −70° C for at least twelve months.

A non-radioactive assay of iothalamate is extremely beneficial for the determination of GFR and renal function. The method described here is simple and rapid, yielding results with minimal sample preparation. The small volume required (25 μl) lends itself to pediatric populations. Except for the initial investment of capital equipment, the assay is relatively inexpensive. The assay is well characterized and consistent over time. Interferences from co-eluting compounds have not been encountered even with dual-wavelength monitoring. Since the eluate is totally aqueous, most drugs tend to be retained by the C_{18} column. One would assume that chromatographic peaks from these compounds would only temporarily offset the baseline and not affect the integrity of the iothalamate or internal standard peak. Loss of resolution between iothalamate and the internal standard peaks or high back-pressure indicated a new column was needed.

This method precludes the necessity of using radionuclide-labelled compounds in the assessment of GFR, and thus eliminates the problems inherent with using them. Urine collection, a potential problem in many cases, is circumvented by utilizing the single-injection method of assessing GFR. This allows for easily repeated evaluations when necessary.

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