Journal of Chromatography, 306 (1984) 89—97
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1947

SIMPLE AND MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF *p*-AMINOHIPPURIC ACID AND IOTHALAMATE IN BIOLOGICAL FLUIDS

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(First received June 15th, 1983; revised manuscript received September 23rd, 1983)

SUMMARY

A simple, rapid and micro high-performance liquid chromatographic method was developed for separate or simultaneous determination of *p*-aminohippuric acid and iothalamate in plasma and urine using *p*-aminobenzoic acid as an internal standard. The method involved deproteinizing samples with two volumes of acetonitrile followed by injection of 5 μl of deproteinized supernatant onto a C_{18} reversed-phase column. The mobile phase contained 3.5% acetonitrile in 0.04% phosphoric acid and flowed at a rate of 1.5 ml/min. The column effluent was monitored by an ultraviolet detector at 254 nm. Retention times for *p*-aminohippuric acid, iothalamate and *p*-aminobenzoic acid were approximately 4.5, 6 and 8 min, respectively. This method requires as little as 5 μl of sample and can be used to measure accurately down to 1 μg/ml *p*-aminohippuric acid and 0.5 μg/ml iothalamate in plasma samples. The coefficients of variation of the assay with or without the use of internal standard were generally low (below 7%). No interferences from endogenous substances or any drugs tested were found.

INTRODUCTION

Determination of glomerular filtration rate and/or renal plasma flow is often useful in pharmacokinetic and pharmacodynamic studies as well as in the diagnosis and treatment of disease [1]. To date, inulin and creatinine appear to have been most commonly employed for estimation of glomerular filtration rate. Potential disadvantages of using the inulin method are the laborious sample preparation, the non-specificity of the colorimetric assay and the need for a sensitive spectrophotometer. Also, in order to achieve steady-state plasma concentrations which can be accurately measured, large doses of inulin are

0378-4347/84/$03.00

usually required for intravenous infusion. In this regard, it seems important to note that the single-injection method with venous sampling might be unsatisfactory, due to potential arterial—venous plasma concentration differences of inulin [2]. Although no administration of exogenous creatinine is required, the use of creatinine clearance for accurate measurement of the glomerular filtration rate has been recently questioned [3]. Contrary to the common notion, creatinine may be extensively secreted and reabsorbed by renal tubules in humans and animals with normal or impaired renal function [4]. Reduction in urine flow or the presence of certain disease states could decrease the creatinine clearance due to enhanced tubular reabsorption, and coadministration of drugs might compete with its tubular secretion. It was also shown that creatinine could be significantly eliminated by non-renal routes [5]. In addition, some commonly used colorimetric assays of creatinine may not be specific, often resulting in a considerable overestimation of plasma levels [6, 7].

The introduction of labeled iothalamate as a substitute for inulin appears very attractive [8, 9]. Its major drawbacks are those associated normally with the use of radioactive compounds, including risks to patients, special storage and handling, and the need for a sensitive detecting instrument. Recently, a highly sensitive high-performance liquid chromatographic (HPLC) method has been reported for the quantitation of low concentrations of non-radioactive iothalamate in plasma and urine [10]. This method is, however, quite tedious, involving four ethyl acetate extractions, and two evaporations of about 10 ml of extract. It also requires 0.5—1.0 ml of sample, and a variable-wavelength ultraviolet (UV) detector (set at 235 nm).

For determination of renal plasma flow, *p*-aminohippuric acid (PAH) has been the most frequently used agent to date [1, 11]. Earlier colorimetric assay methods based on the color reaction between PAH and a reagent have been known to be non-specific and relatively time-consuming [12]. Several HPLC methods have been published in recent years. The method reported by Brown et al. [13] employed direct injection of biological samples which might be detrimental to the column. Their HPLC system had an unusually unstable baseline, necessitating the preparation of buffer solution 12—24 h in advance and storage of mobile phase in the pump. The sensitive method of Shoup and Kissinger [12] employed direct injection of biological samples diluted with distilled water, and a less commonly available and less easy-to-operate electrochemical detector. The method of Gloff et al. described in an abstract [14] appeared adequate for most routine analyses. They employed a high percentage (50%) of acetonitrile in the mobile phase, and the method had a coefficient of variation up to about 14%. A capillary gas chromatographic method reported recently [15] used a special nitrogen—phosphorus detector. The assay also required 1 ml of plasma, ether extraction and derivatization.

The purpose of this paper is to report a simple, rapid and micro HPLC method for separate or simultaneous quantitation of iothalamate and PAH in biological fluids using a fixed-wavelength UV detector.

EXPERIMENTAL

Reagents and standards

PAH (as a sodium salt) was purchased from Sigma (St. Louis, MO, U.S.A.), iothalamate (as an acid form) was purchased from Mallinkrodt (St. Louis, MO, U.S.A.) and *p*-aminobenzoic acid (PABA) from Mann Research Lab. (New York, NY, U.S.A.). Phosphoric acid (85%) and HPLC grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Standard stock solutions of PAH, iothalamate and PABA were prepared in distilled water at concentrations of 0.5—2 mg/ml and stored at 4°C in a refrigerator. All the solutions were used within a few weeks because a slight decomposition of PAH was found after 5—6 weeks of storage.

HPLC instrumentation

The HPLC system consisted of a solvent delivery pump (Model 110A), a fixed-wavelength detector with a 254-nm filter (Model 160) from Beckman Instruments (Berkeley, CA, U.S.A.), a syringe loading sample injector (Model 7215, Rheodyne, Cotati, CA, U.S.A.) and a 30-cm μBondapak C_{18} reversed-phase column (particle size 10 μm, I.D. 4.1 mm) from Alltech Associates (Deerfield, IL, U.S.A.). The output from the detector was connected to a 10-mV potentiometric 25-cm recorder (Linear Instruments, Irvine, CA, U.S.A.). A chart speed of 10 cm/h was employed for routine analysis [16]. The detector was set at 0.002—0.01 and 0.005—0.05 a.u.f.s. for plasma and urine analyses, respectively.

Mobile-phase preparation

The mobile phase was prepared by mixing 3.5 parts of acetonitrile with 96.5 parts of 0.04% phosphoric acid solution (pH 2.5 ± 0.05). A flow-rate of 1.5 ml/min resulted in a pump pressure of approximately 84 bar. The optimal percentage of acetonitrile might vary slightly with the column.

Standard curves

Aliquots of 100 μl of pooled human plasma spiked with various known quantities of stock solutions of PAH, iothalamate, and PABA were pipetted into 100 × 13 mm screw-capped culture tubes followed by the addition of 200 μl of acetonitrile. After capping, each tube was vortexed for a few seconds and centrifuged at 800 *g* for 5 min. The clear supernatant, 5 μl, was then injected onto the column. Urine samples were prepared in the same manner. The concentrations of PAH and iothalamate ranged from 2.5 to 50 μg/ml in plasma, and 10 to 100 μg/ml in urine. The internal standard concentrations were 5 or 25 μg/ml and 25 or 50 μg/ml in plasma and urine, respectively.

The peak heights, measured with the assistance of a micrometer (Dial Caliper from Manostat, NY, U.S.A.), were used for quantitation and the ratios of peak heights of PAH or iothalamate to those of the internal standard were used to construct the standard curves.

Reproducibility study

Six replicate analyses of pooled human plasma containing 15 or 30 μg/ml

PAH and iothalamate were analyzed according to the method described earlier.

Recovery study

The recovery was assessed by comparing the absolute peak height of PAH or iothalamate from plasma and urine samples to those obtained by direct injection of aqueous solutions at concentrations of 15 and 30 μg/ml for each compound.

Drug interference study

Many drugs and a potential metabolite of PAH, N-acetyl-*p*-aminohippuric acid (N-acetyl-PAH) [17, 18], were tested for interference with the assay by injecting stock solutions of these compounds onto the column. The drugs tested included those which may produce nephrotoxicity [19] (e.g. *p*-aminosalicylic acid, isoniazid, kanamycin, gentamycin, tobramycin, amikacin, trimethadione) and those which can interfere with PAH determination using colorimetric methods [12] (e.g. methyldopa, sulfathiazole, etc.). Acetaminophen, aspirin, propranolol, acetazolamide, tolazamide, hydralazine and methotrexate were also tested. The N-acetyl-PAH was prepared according to the method of Newman et al. [18].

Preliminary study in dog.

A 5% sterile iothalamate solution was prepared by dissolving an equimolar ratio of iothalamate and sodium hydroxide in water followed by autoclaving. The sterile PAH solution (1% w/v in 0.9% sodium chloride) was prepared by filtering through a 0.2-μm filter unit (Millex-FG from Millipore, Bedford, MA, U.S.A.). Aliquots of each solution in 0.9% sodium chloride (equivalent to 180.5 mg of PAH and 219.2 mg of iothalamate) were then simultaneously infused into the cephalic vein of a male mongrel dog (10.1 kg) over 40 min. Venous blood samples were collected at appropriate times and were immediately centrifuged at 800 *g* for 5 min to separate the plasma. Urine samples were collected at 30-min intervals. All the samples were stored frozen until analyzed (within one week). Blank samples of plasma or urine were obtained before drug administration and processed in a similar manner. Standard curves were constructed using blank samples from the same dog.

RESULTS AND DISCUSSION

Chromatograms from typical blank human plasma and urine, and from those containing known concentrations of PAH, iothalamate, and PABA are shown in Fig. 1, while those from plasma and urine samples obtained from the dog given 180.5 mg of PAH and 219.2 mg of iothalamate are depicted in Fig. 2. The retention times of PAH, iothalamate and PABA were approximately 4.5, 6 and 8 min, respectively. No interfering peaks were found in various plasma and urine blanks examined. Although there was an endogenous peak found between the iothalamate and PABA peaks in dog plasma, it did not affect the present assay. Another endogenous peak appeared from both human and dog plasma with a retention time of 18—19 min. In order to save time between analyses, one can inject the second sample right after the appearance of the PABA peak from the first sample.

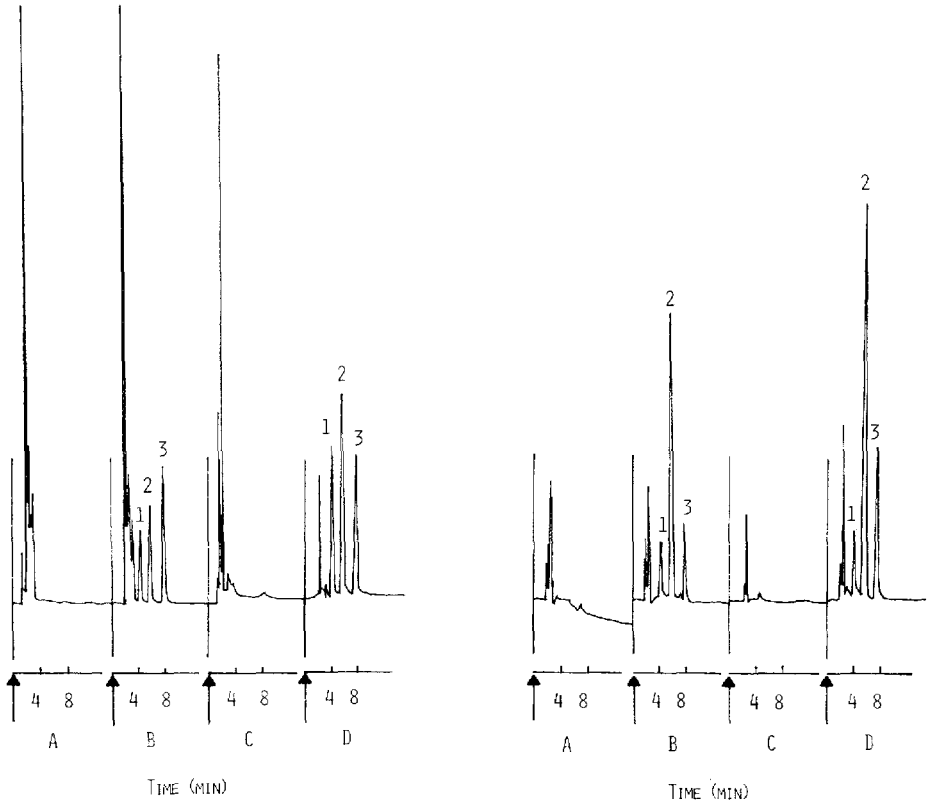

Fig. 1. Chromatograms of: (A) blank human plasma; (B) human plasma spiked with 15 μg/ml PAH, iothalamate and 30 μg/ml PABA; (C) blank human urine; (D) human urine spiked with 30 μg/ml PAH, iothalamate and PABA. Peaks: 1 = PAH, 2 = iothalamate, 3 = PABA. Sensitivity setting: 0.01 a.u.f.s. The arrows mark the points of injections.

Fig. 2. Chromatograms of: (A) blank dog plasma; (B) dog plasma obtained after 1 h infusion of 180.5 mg of PAH and 219.2 mg of iothalamate; (C) blank dog urine; (D) dog urine collected after 3 h of infusion. Peaks: 1 = PAH, 2 = iothalamate, 3 = PABA. Sensitivity setting: 0.01 a.u.f.s. for A and B, 0.025 a.u.f.s. for C and D. The arrows mark the points of injections.

The pH of the mobile phase was found to play an important role in peak resolution. At pH values higher than 2.6, iothalamate was eluted faster and interfered with PAH, whereas at lower pH values (less than 2.4), iothalamate eluted later and interfered with PABA.

The μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size) from Waters Assoc. also gave adequate peak separation and similar retention times for the three compounds under the current HPLC conditions. However, the C_{18} column (Partisil PXS 10/25 ODS, Cat. No. IF 2494) from Whatman (Clifton, NJ, U.S.A.) did not give a satisfactory result under these conditions.

The standard curves for human plasma and urine samples were found to be linear over the concentration range studied. This is illustrated by the low coefficients of variation of response factors as shown in Tables I and II. Without the use of internal standard, the coefficients of variation for plasma and urine were also low (Tables I and II). Based on a signal-to-noise ratio of 3,

TABLE I

RESPONSE FACTORS OF PAH AND IOTHALAMATE IN HUMAN PLASMA

Spiked plasma concentration (μg/ml)	With internal standard (based on concentration of 5 μg/ml)				Without internal standard (based on sensitivity setting of 0.002 a.u.f.s.)	
	Peak height ratio*		Response factor I**		Response factor II***	
	PAH	Iothalamate	PAH	Iothalamate	PAH	Iothalamate
2.5	0.52	0.80	0.20	0.32	0.54	0.83
5.0	0.96	1.47	0.19	0.29	0.56	0.86
10.0	2.09	3.19	0.21	0.32	0.54	0.83
25.0	5.43	7.50	0.22	0.30	0.56	0.77
50.0	10.95	15.20	0.22	0.30	0.55	0.77
Mean ± S.D.			0.21 ± 0.012	0.31 ± 0.012	0.55 ± 0.01	0.81 ± 0.039
C.V. (%)			5.62	3.74	1.84	4.80

*Peak height ratio = ratio of peak height of the compound to that of the internal standard.
**Response factor I = peak height ratio divided by the concentration (μg/ml) of the compound.
***Response factor II = peak height (cm) of the compound divided by its concentration (μg/ml).

TABLE II

RESPONSE FACTORS OF PAH AND IOTHALAMATE IN HUMAN URINE

Spiked urine concentration (μg/ml)	With internal standard (based on concentration of 20 μg/ml)				Without internal standard (based on sensitivity setting of 0.005 a.u.f.s.)	
	Peak height ratio		Response factor I (ml/μg)		Response factor II (cm/μg/ml)	
	PAH	Iothalamate	PAH	Iothalamate	PAH	Iothalamate
10.0	0.62	0.84	0.062	0.084	0.19	0.26
20.0	1.24	1.61	0.062	0.080	0.18	0.24
40.0	2.50	3.27	0.063	0.082	0.18	0.23
60.0	3.71	4.81	0.062	0.080	0.17	0.22
100.0	6.20	8.12	0.062	0.081	0.17	0.23
Mean ± S.D.			0.062 ± 0.0003	0.081 ± 0.001	0.18 ± 0.009	0.24 ± 0.015
C.V. (%)			1.74	0.47	4.97	6.53

the detection limits of the present assay for plasma samples were found to be 1 μg/ml for PAH and 0.5 μg/ml for iothalamate.

The intra-day coefficients of variation were 1.60% and 3.79% for PAH, and 1.59% and 0.74% for iothalamate at the concentrations of 15 μg/ml and 30 μg/ml, respectively. The inter-day coefficients of variation for the analysis of the same plasma samples on four days over a period of one week were 2.75% and 7.20% for PAH, while for iothalamate the values were 5.5% and 5.95% at the concentrations of 5 and 25 μg/ml, respectively.

At concentrations of 15 and 30 μg/ml, the percentages of recovery of iothalamate were 94% for plasma and 95—100% for urine, while those of PAH were virtually 100% for both plasma and urine. These high recoveries resulted from the simple one-step deproteinization with two volumes of acetonitrile. The uniqueness of acetonitrile as a simple and effective deproteinizing agent has been extensively studied earlier [20]. Use of 20% perchloric acid to precipitate proteins in plasma samples was not found to give better results

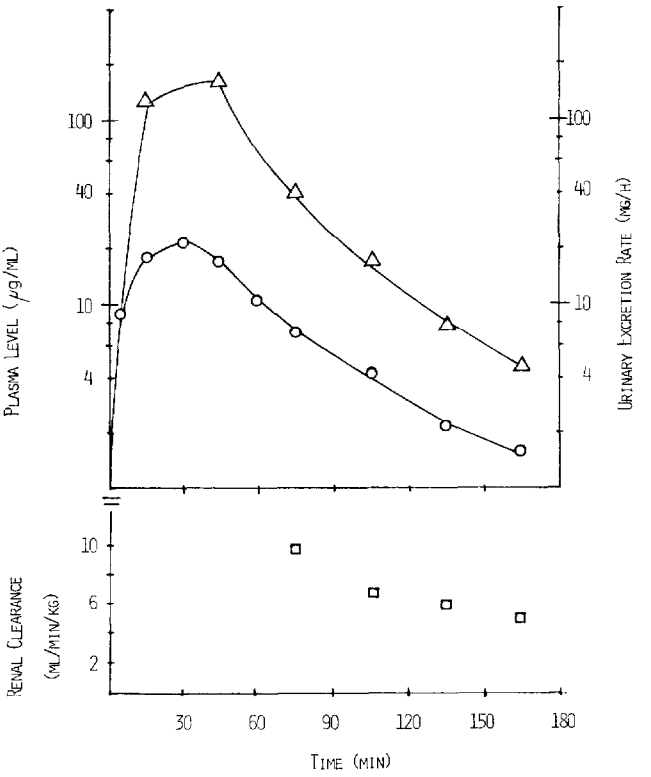

Fig. 3. Plasma concentration profile (○) and urinary excretion rate profile (△) of PAH in the dog following 40 min infusion of 180.5 mg of PAH and 219.2 mg of iothalamate. Renal clearance of PAH from the dog (□) was obtained by dividing urinary excretion rate by plasma concentration at the midpoint of urine collection interval.

than using acetonitrile. With the same volume of injection (5 μl), it resulted in a higher response and more interference. It is obvious that, when necessary, sample sizes smaller than 0.1 ml can be used for quantitation.

Peak splitting was found when the volume of injection was more than 5 μl under the present HPLC conditions.

The results of the drug interference study showed that none of the drugs tested would affect the assay, with the exception of hydralazine which might coelute with the internal standard. N-Acetyl-PAH, a metabolite of PAH in man [17, 18], did not interfere with the assay. Its retention time was about 30 min.

The plasma and urine profiles of both compounds from the preliminary dog study together with their renal clearances (determined by midpoint method) are shown in Figs. 3 and 4. In spite of the venous data employed in the present study [2], the gradual decrease in PAH renal clearance during the apparent terminal phase was evident; this appeared to be different from the result in dogs [18] but consistent with that in man reported earlier [18]. The total urinary recoveries estimated were 94.3% and 87.5% for PAH and iothalamate, respectively. No N-acetyl-PAH was found in either plasma or urine samples, which is in agreement with the previous studies [17, 18]. PABA, which was also reported to be a possible metabolite of PAH in man [21], was not

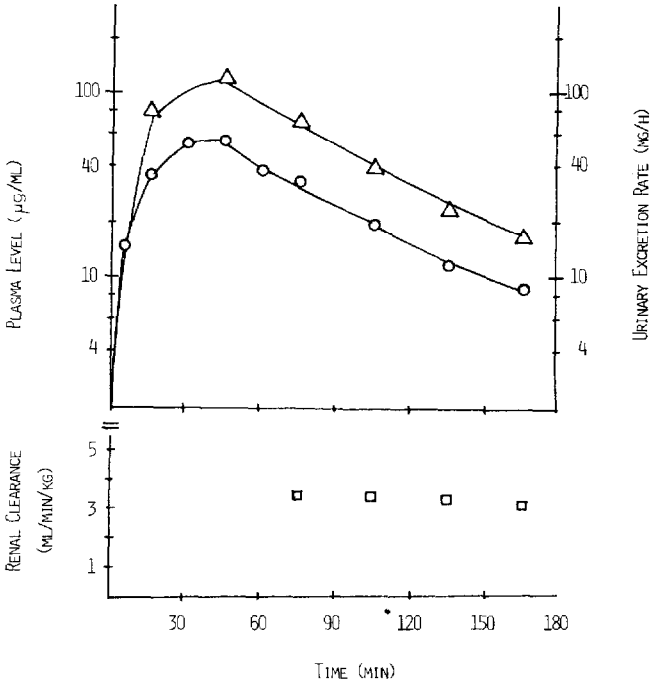

Fig. 4. Plasma concentration profile (○) and urinary excretion rate profile (△) of iothalamate in the dog following 40 min infusion of 180.5 mg of PAH and 219.2 mg of iothalamate. Renal clearance of iothalamate from the dog (□) was obtained by dividing urinary excretion rate by plasma concentration at the midpoint of urine collection interval.

detected. Even if PABA cannot be used as an internal standard due to its presence as a metabolite, the present HPLC method may still be satisfactory. When necessary, an alternative internal standard such as hydralazine might be employed.

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